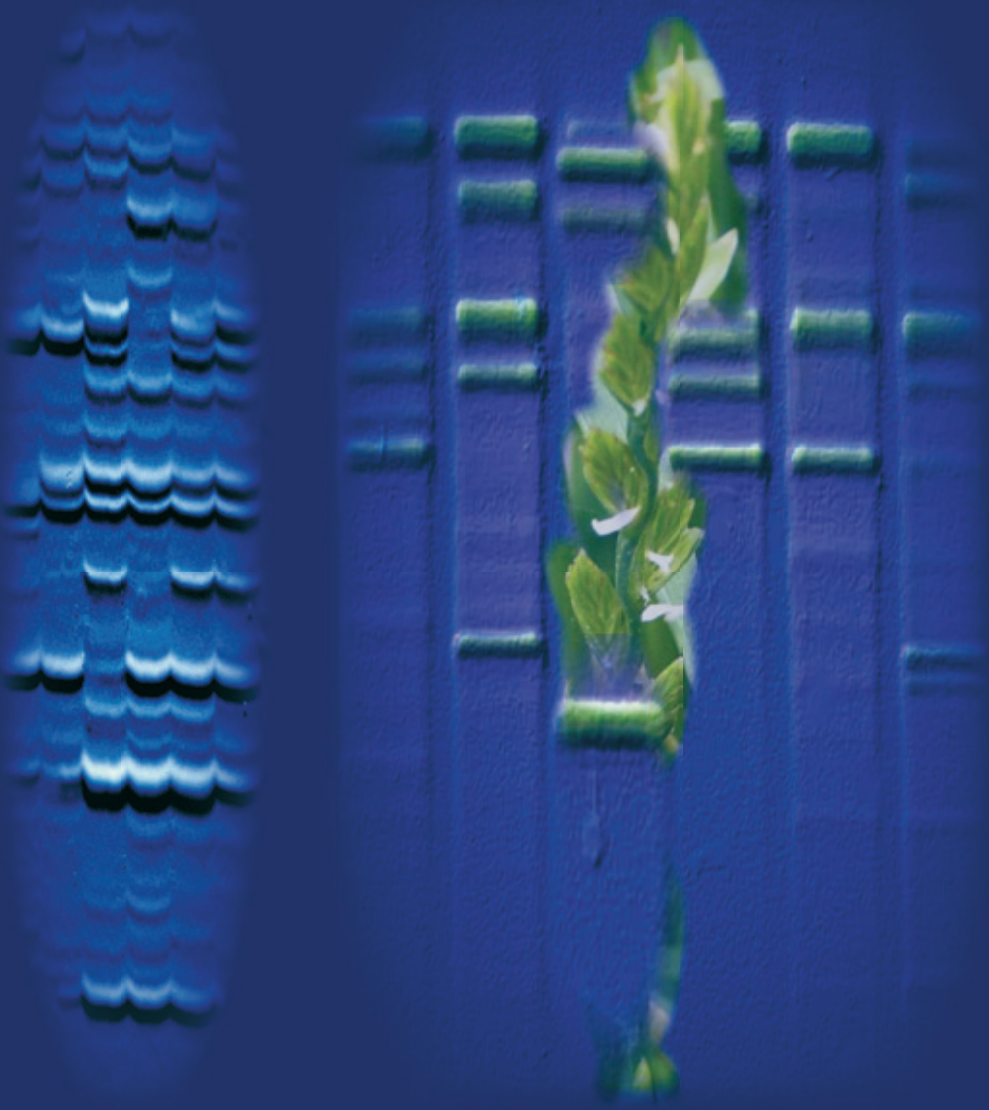
Molecular evolution of the genus *Lolium* L.

Kornelia Polok



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„The species is the principal unit of evolution and it is impossible to write about evolution, without having a sound understanding of the meaning of biological species"

(Mayr 1957)

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CONTENTS

1. INTRODUCTION

1.1. MORPHOLOGY, BIOLOGY AND DISTRIBUTION OF THE GENUS *LOLIUM*

The name of the genus *Lolium* (ryegrasses) first was mentioned in Virgil's Georgics. It is an old Latin name given for a troublesome weed, *Lolium* temulentum. The genus consists of about eight recognised species that are mostly annual herbs (Figure 1.1). *Lolium* plants can be as short as 10 cm but also quite high culms are observed (150 cm). A quick check to determine if the grass is *Lolium* is to see if there are two glumes. In the single spikelike flowerhead the spikelets are attached edgewise directly to the spike axis and along this edge there is no glume. Plants are bisexual, with bisexual spikelets and hermaphrodite florets. The three species, *L. multiflorum*, *L. perenne* and *L. rigidum* are wind-pollinated, out-breeding (allogamous) species, the other four, *L. loliaceum*, *L. persicum*, *L. remotum*, and *L. temulentum* are self-pollinated, inbreeding species (autogamous), while *L. canariense* shows a moderate level of cross fertilization. All species are diploids with 2n=14, but due to breeding activities tetraploid cultivars are available. The mean haploid nuclear DNA content is 5.0 pg and ranges from 2.2 to 6.9 pg (Terrel 1968; Watson and Dallwitz 1999).

The genus *Lolium* consists of Old World Species. It is native to Europe, temperate Asia and North Africa although most species have been widely distributed around the temperate areas of the world. They are common as weeds or cultivated fodder grasses in North America, Australasia and Pacific region (Table 1.1). The only exception is *L. canariense* that is endemic to the Canary Islands. The widest distribution is observed for two allogamous species, *L. multiflorum* and *L. perenne* and autogamous *L. temulentum* including almost the whole Europe, the significant part of Asia, Africa, Australasia and both Americas. However, *L. multiflorum* also known as Italian ryegrass grows the best in Mediterranean climates. On opposite, *L. perenne* is found growing further north than any of the other species and it is best adapted to cool, moist climates where winter kill is not a problem. It is interesting to note that this species is more sensitive to temperature extremes and drought than annual ryegrass.

The members of the genus *Lolium* are adapted to mild, humid climate but they are tolerant of a wide range of soils and climates. They grow best on rather heavy, fertile, moist soils, but do well on lighter soils with sufficient moisture. The optimum soil pH is between 5.5 and 7.5 although both more acidic (pH 5.1) and more alkaline (pH 8.4) soils are also tolerated. They perform less than optimum during a drought or periods of extended low or high temperature. Some species tolerate long periods of flooding ("Species: Ryegrass" 2006).

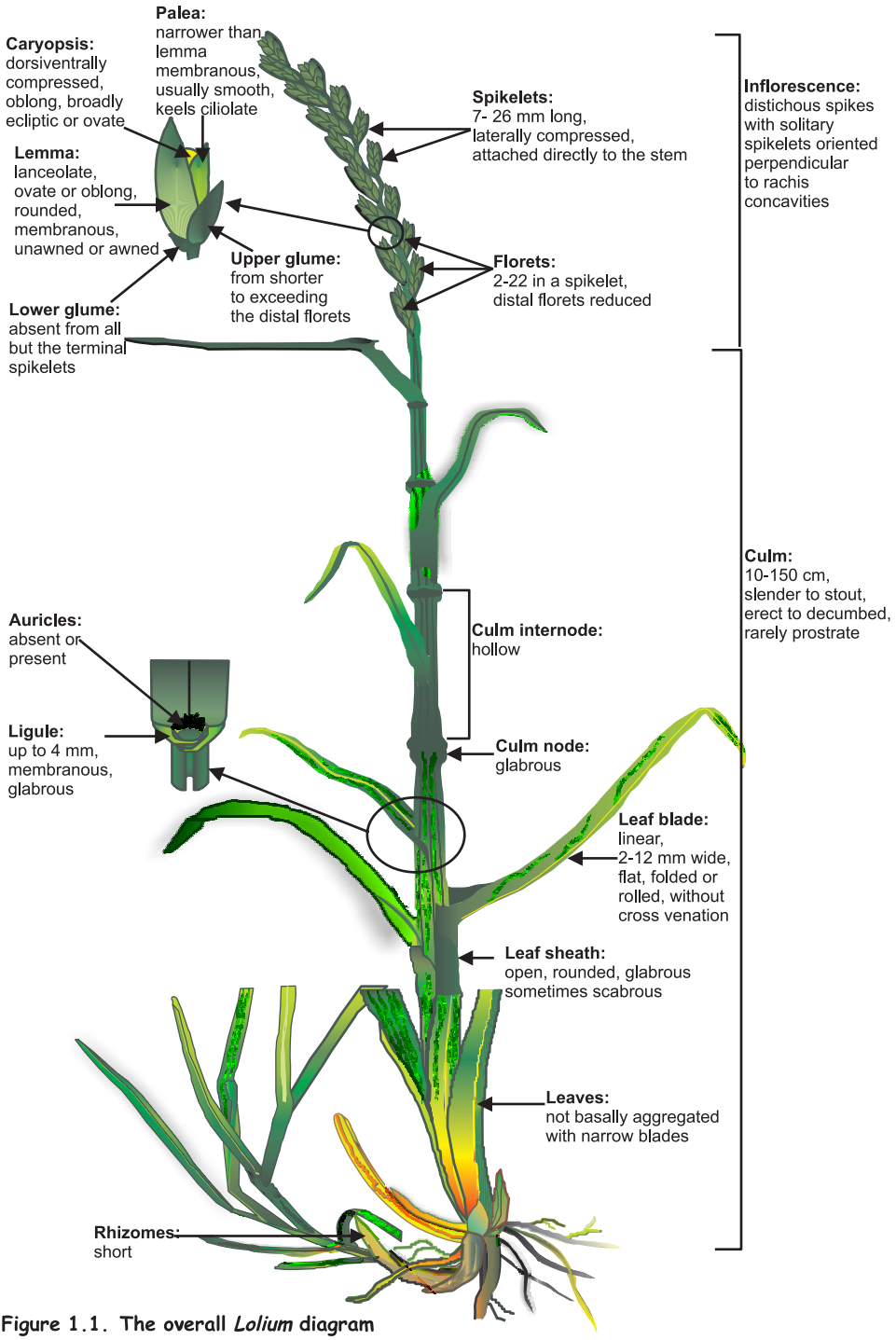


Figure 1.1. The overall *Lolium* diagram

Table 1.1. Distribution of the genus *Lolium*

Species	Native	Introduced
L. canariense	**Africa**: Macaronesia, endemic to the Canary Islands	-
L. loliaceum	**Europe:** northern, central, southwestern; **Asia-temperate:** Soviet Middle Asia, Caucasus, western Asia; **Asia-tropical:** India	**Australasia:** Australia
L. persicum	**Europe:** eastern; **Asia-temperate:** Soviet far east, Soviet Middle Asia, Caucasus, western Asia, Arabia, China; **Asia-tropical:** India	**North America:** western Canada, eastern Canada, north-central USA
L. remotum	**Europe**: northern, central, southwestern, eastern; **Africa**: north, Macaronesia; **Asia-temperate**: Siberia, Soviet far east	**Australasia:** Australia
L. temulentum	**Europe**: northern, central, southwestern, southeastern, eastern; **Africa**: north, Macaronesia, northeast tropical, east tropical, southern tropical, south and western Indian ocean; **Asia-temperate**: Siberia, Soviet far east, Soviet Middle Asia, Caucasus, western Asia, Arabia, eastern Asia; **Asia-tropical:** India,	**Australasia:** Australia and New Zealand; **Pacific:** north-central; **North America:** Subarctic, western Canada, eastern Canada, northwest USA, northeast USA, southwest USA, south-central USA, Mexico; **South America:** Mesoamericana, Caribbean, northern South America, western South America, Brazil, southern South America; **Antarctic**: Subantarctic islands
L. multiflorum	**Europe**: northern, central, southwestern, southeastern, eastern; **Africa**: north, Macaronesia, northeast tropical, east tropical, southern tropical, south; **Asia-temperate**: Soviet far east, Soviet Middle Asia, Caucasus, western Asia, Arabia, China, eastern Asia; **Asia-tropical:** India	**Australasia:** Australia and New Zealand; **Pacific:** north-central; **North America:** Subarctic, western Canada, eastern Canada, northwest USA, north-central USA, northeast USA, southwest USA, south-central USA, southeast USA, Mexico; **South America:** Mesoamericana, Caribbean, western South America, Brazil, southern South America; **Antarctic**: Subantarctic islands
L. perenne	**Europe**: northern, central, southwestern, southeastern, eastern; **Africa**: north, Macaronesia, northeast tropical, southern tropical, south; **Asia-temperate**: Siberia, Soviet Middle Asia, Caucasus, western Asia, Arabia, China, eastern Asia; **Asia-tropical:** India, Indo-China	**Australasia:** Australia and New Zealand; **Pacific:** south-central, north-central; **North America:** Subarctic, western Canada, eastern Canada, northwest USA, north-central USA, northeast USA, southwest USA, south-central USA, southeast USA, Mexic;, **South America:** Mesoamericana, western South America, Brazil, southern South America; **Antarctic**: Subantarctic islands
L. rigidum	**Europe**: central, southwestern, southeastern, eastern; **Africa**: north, Macaronesia, south; **Asia-temperate**: Soviet far east, Soviet Middle Asia, Caucasus, western Asia, Arabia, eastern Asia; **Asia-tropical:** India	**Australasia:** Australia and New Zealand; **South America:** southern South America; **Antarctic**: Subantarctic islands

Summarized on the basis of Clayton et al. 2006

1.2. ECONOMIC IMPORTANCE OF THE GENUS *LOLIUM*

The genus *Lolium* is probably the most highly domesticated of all herbage grasses. In general all ryegrasses are significant weed species, some are cultivated and they can be casually used in folk medicine. The association of *L. temulentum* with corn and *L. remotum* with flax may be as remote as 2000 years ago and even annual *L. loliaceum* has been found to be connected with human activities in certain areas of Australia and America (Jenkin 1959). The evil reputation of *L. temulentum* is of very ancient date. The name “Darnel” is of French origin and means stupefied because of symptoms such as confusion in mind, very great depression, paralysis, tremors and convulsions observed in people poisoned by eating meal containing *L. temulentum* seeds. However, the poisonous properties of Darnel are now generally believed to be due to an ergot. This view is supported by the fact that the poisonings have been most frequently observed in low, wet areas during wet seasons. *L. temulentum* is

occasionally used in folk medicine to treat headache, rheumatism and externally in cases of skin diseases (Clarke 2000).

L. persicum, Persian darnel belongs to one of the most serious weeds in North America, where it has been recently introduced. At high densities it could cause wheat yield loss up to 80%. Because of short stature Persian darnel is difficult to see in maturing grain field and therefore, it often grows unchecked allowing the population to build until it is too late. In addition, *L. persicum* has developed resistance to herbicides as well as it is able to produce seeds following herbicide applications. Such attributes as early emergence and rapid development make the Persian darnel especially competitive weed in dryland wheat and oat production (Bussan and Trainor 2001).

All allogamous species can be cultivated but they can become serious weeds when they escape from fields. Originating at least partially from cultivars, they often display a propensity to evolve resistance to herbicides. In Australia *L. rigidum* used to be a useful agricultural plant in areas with a short winter rainy season. But at present it is established in large numbers over the 40 million ha that embrace the southern Australian winter cropping and pasture region. Populations of this species display resistance to most of the major herbicides (Powles et al. 1998).

The two most important ryegrass species are Italian ryegrass (*Lolium multiflorum*) and perennial ryegrass (*Lolium perenne*), which widely grow as forage and cover crops as well as turf grasses in Europe, North and South America, New *Zea*land and Australia. They establish rapidly, have a long growing season and high yield under favorable environment. Italian ryegrass has a bunch-type growth and flowers in day lengths greater than 11 hours. There are no winter or cold weather requirements to flower and therefore, it will flower throughout the summer. Perennial ryegrass is also a bunch-type grass but unlike Italian ryegrass, it requires a dormancy period of low temperatures to induce flowering. Cultivars of both ryegrasses are primarily diploids but numerous tetraploid cultivars for forage uses have been developed. They have higher digestibility and grazing preference due to a higher percentage of sugars ("Species: *Lolium perenne*" 2006).

Both ryegrasses are considered to be high quality forage, and their high digestibility together with high crude protein content during vegetative growth make them suitable for all classes of livestock and most wild ruminants. Italian ryegrass produces high yields and maintains productivity through mid-summer better than most other cool season grasses and thus, it provides high quality grazing for dairy cattle. Fresh early bloom aerial portion of Italian ryegrass contain 55% of nitrogen-free extract, 30% of crude fiber and almost 6% of crude protein (Carey 1999). For early sowing, the true annual type of *L. multiflorum* is used - var. *westerworldicum* characterized by very high vigour and high yields. Annual Westerwold ryegrass was probably introduced as an ecotype selected from Italian ryegrass in the beginning of the XX century (Jenkin 1959).

Perennial ryegrass is considered the premier quality pasture grass throughout the world, having higher digestibility than other temperate perennial grass species. Depending on the developmental stage the average in vitro digestibility ranges from 71% for mature plants up to 88% during vegetative growth. The crude protein content increases with nitrogen soil fertility and can reach as much as 18% ("Species: *Lolium perenne*" 2006). The grass is also a good source of carotene (4.8 mg/100 g). It contains free fructose, fructosan, mannitol,

a complex mixture of oligosaccharides, oxalic-, citric-, malic-, and shikimic acids, glycerides, and a wax containing hexacosanol. Seeds are comparable to oats in nutritive value; they contain a prolamine and a gluten similar to wheat gluten (Duke 1983). It is suitable for all classes of livestock, especially those with high nutrient requirements such as young, growing animals and lactating dairy cows. *L. perenne* is often harvested for silage and hay except high rainfall and humidity areas (Hannaway et al. 1999).

High growth rates make perennial ryegrass valued for use in nutrient recycling system. It can utilize up to 450 kg of N/ha from livestock manure or biosolids. This ability results in high quality forage and protects groundwater from contamination (Hannaway et al. 1999).

The use of ryegrasses for turf has exploded in the last thirty years. Italian ryegrass predominates in homeowner lawns while perennial ryegrass is preferred for parks, sport turf and golf courses. *L. perenne* is especially popular in warmer climates where it remains green all winter. Perennial ryegrass is well suited to soil conservation on badly eroded mine spoils or severely burned areas. Its extensive and fibrous root system makes it effective for reducing soil erosion. It provides rapid cover and allows longer-lived or more winter-hardy species to become established (Hannaway et al. 1999). *L. hybridum* was first introduced about 20 years ago in order to combine some of the best qualities of *L. perenne* with some characters of *L. multiflorum*. The resultant plants have attributes of both species. It is less winter hardy but higher yielding that perennial ryegrass. It can be used for temporary turf and forage production.

Because of excellent features as agricultural and recreational species, the ryegrasses have become the dominant component of grasslands in temperate areas of the world. The close contact with major human population centers together with the presence of the allergenic proteins in the pollen, lead these species to be one of the most important causes of the seasonal asthma and hay fewer. A range of allergens have been identified in ryegrass pollen which are complex mixture of various proteins. At least 17 allergenic proteins have been identified in perennial ryegrass pollen (Spangenberg et al. 2006). The major allergen, Lol pI is a glycoprotein of about 27 kDa to which 85-90% of ryegrass allergic patients are sensitive. Lol p II is the second major allergen, to which 45% of ryegrass allergic patients are reactive (Sidoli et al. 1993).

1.3. ORIGIN AND POSITION OF *LOLIUM* WITHIN POACEAE

For years there has been a great deal of disagreement over the origin and classification of ryegrasses. What is the common ancestor of the genus, or what is the position within the grass family, or what are relationships between the *Lolium* species? A major limitation of these considerations is the lack of data about natural distribution. The genus has been accompanying man for ages that makes the natural populations and cultivars uniform. The earliest records of its cultivation arise from the 13th-14th century (Jenkin 1959). All ryegrasses are supposed to originate from the Mediterranean basin wherefrom migrated northwards in Europe. Because they are known as weeds or cultivated crops they probably evolved in close association with primitive agriculture (Charmet and Balfourier 1994).

On the basis of embryo type, leaf anatomy, and chromosome base number the genus *Lolium* is classified within the family Poaceae, and the subfamily Pooideae (Figure 1.2). The subfamily Pooideae is one of the major lineages of the grasses that diverged together with Bambusoideae and Ehrhartoideae during the first major radiation of the grasses about 46 million years ago (Kellogg 1998; Gaut 2002). These three subfamilies are often referred as BEP clade. According to the most recent classifications based on chloroplast restriction site variation, sequences of different chloroplast genes, some nuclear genes and internal transcribed region (ITS), the Pooideae contains 12 tribes. The four from six more recently diverged tribes "Triticeae, Bromeae, Poeae and Aveneae are united by having large chromosomes of x=7 and they are often classified as "Core Pooids". The "Core Pooids" are resolved into two clades, first consisted of two closely related monophyletic tribes Poeae and Aveneae while the second one contains Bromeae and Triticeae (Catalan et al. 1997; Kellog 1998; Gaut 2001).

The close association of the genus *Lolium* with agriculture in addition to the morphology of inflorescences that, at first glance, are like the spikes of barley or rye persuaded early researchers to the classification of ryegrasses within the tribe Triticeae (syn. Hordeae, Hordeeae). Referring to *Lolium* systematics Jenkin (1959) wrote, "Spicate inflorescences placed the genus *Lolium* easily and naturally into this tribe and even the inflorescences are branched, the branching is a kind peculiar to *Lolium*". On the other hand, Hubbard (1948) argued that the spicate inflorescence of *Lolium* might have a separate evolutionary origin. Considering spikelets, starch grains in the seeds and crossability between *Lolium* and *Festuca* he was the first who classified ryegrasses in the tribe Festuceae. Further cytological and molecular studies have supported the close relationships between both genera and all modern classifications include *Lolium* and *Festuca* into the tribe Poeae (syn. Festuceae) together with the other grasses such as Poa, Dactylis. The linkage between *Festuca* and *Lolium* is supported by the fact that *F. arundinacea* and *L. perenne* share a two-codon insertion in the chloroplast *ndhF* gene relative to the other Pooideae (Catalan et al. 1997). Of particular importance are the relationships between *Lolium* and broad-leaved fescues including *F. pratensis* (meadow fescue), and *F. arundinacea* (tall fescue) i.e., those historically placed in *Festuca* subg. *Schedonorus*. Charmet et al. (1997) reported that tall fescue and meadow fescue were the most similar to all ryegrasses on their UPGMA dendrograms based on cpDNA restriction sites and ITS (Internal Transcribed Spacer). They estimated the divergence of *Festuca* from *Poa trivialis* about 13 million years ago, and then broad-leaved and fine-leaved fescues split about 9 million years ago.

The genus *Lolium* is considered to be more recent than *Festuca*, because it produced no polyploids and has a restricted distribution (Bulińska-Radomska and Lester 1988). The small number of species within *Lolium* (about 8 species) in comparison with *Festuca* (about 400 species) has also been taken as evidence of a more recent origin of the former. However questions inevitable arise if *Lolium* originated from *Festuca* or both genera had a common ancestor. Essad (1962) suggested that *Lolium* was derived from *Festuca* through the transformation of a panicle into a spike. In the course of grass evolution, spike and panicle form of inflorescence have frequently arisen from each other. Likewise, restriction sites in cpDNA show that the genus *Lolium* has diverged from *Festuca* about 2 million years ago (Charmet et al. 1997), long after the differentiation of fine-leaved fescues (subg. *Festuca*).

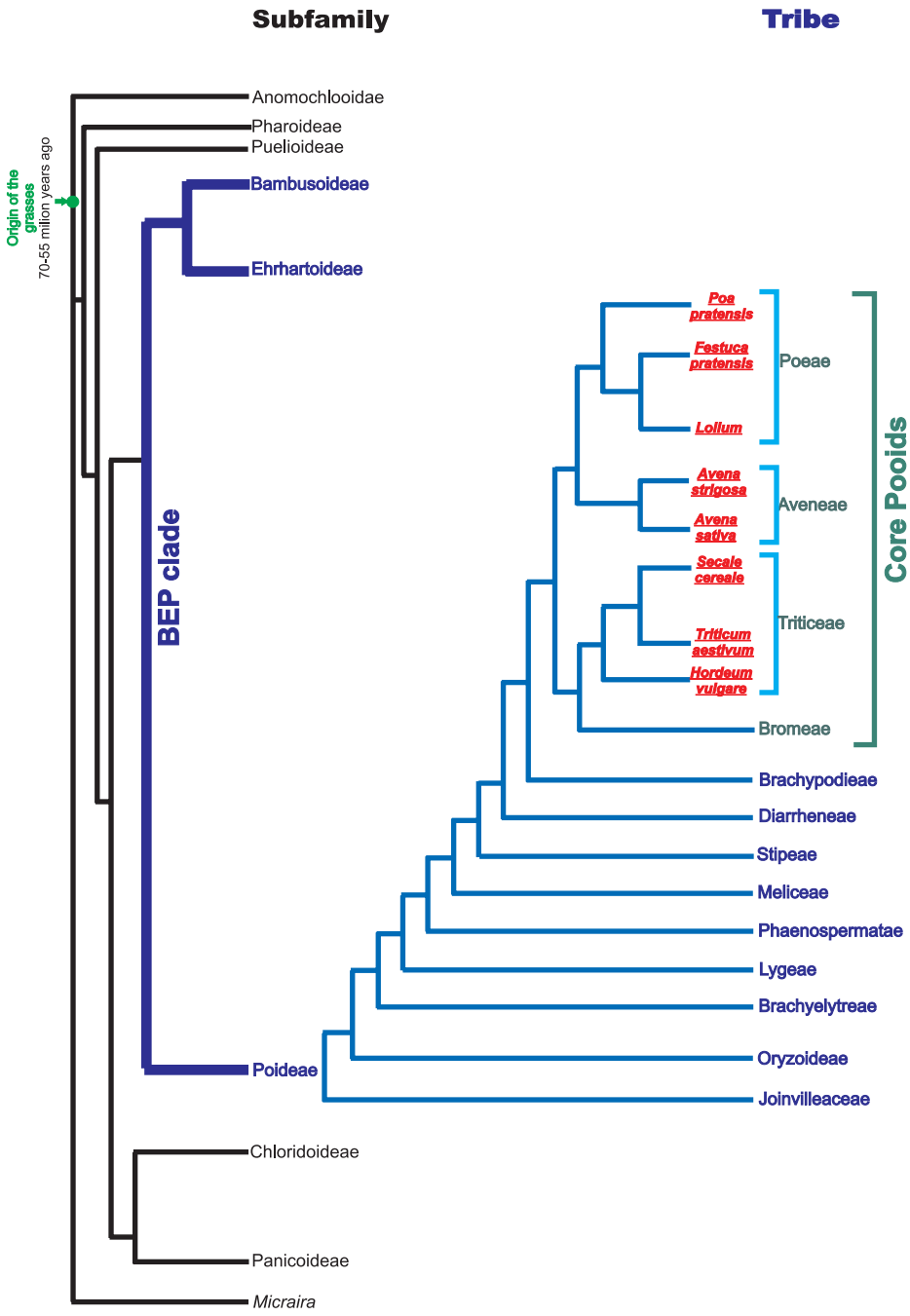


Figure 1.2. Phylogeny of Poaceae based on combined data (Catalan et al. 1997; Kellog 1998; 2001)

Heavy lines indicate BEP clade, species included in the present analysis are underlined

The evidences from ITS and *trnL*-F sequences might weight in favour of the *Lolium* evolution from a perennial, European *Schedonorus* ancestor (Catalan et al. 2004). On the other hand, experimental data from protein studies (Bulińska-Radomska and Lester 1988) and patterns of cross-hybridization (Xu et al. 1992) make more plausible the Stebbins (1958) hypothesis that both genera had a common ancestral form with a base chromosome number x=7. Notwithstanding these difficulties in finding any ancestral form, the ease of interspecific hybridization between *Lolium* and *Festuca* from the subgenus *Schedonorus* and similarity of large number of morphological characters convinced some taxonomists for classification of *Schedonorus* within the genus *Lolium* (Darbyshire 1993; Stammers et al. 1995). Under this taxonomic scheme *Lolium* is not any longer a small genus with only diploid species but it consists from a number of species with various ploidy levels. The incorporation of *Schedonorus* into the genus *Lolium* suggests that polyploidy has played an important role in its evolution (Craven et al. 2005). If we assume, as most grass taxonomists will argue, that diploidy is the ancestral condition, then all *Lolium* species should be regarded as more ancient than all polyploid fescues from *Schedonorus*. It should be noticed, however, that the above classification is not widely accepted. One emerging consensus seems to be that the relationship between *Festuca* and *Lolium* is difficult to deduce conclusively on the basis of available molecular data.

1.4. TAXONOMY OF THE GENUS *LOLIUM*

Another challenge underlying the genus *Lolium* is in finding the progenitor and defining exactly a kind of species differentiation. In principle, monophyly of *Lolium* has been accepted but it is still a source of contention either *L. perenne* or *L. rigidum* is the common ancestor of the genus. Based on total nuclear DNA content, which increases from *L. perenne* (4.2 pg) to *L. temulentum* (6.2 pg), Thomas (1981) speculated that speciation of *Lolium* might have involved isolates of *L. perenne*. Protein studies suggest as well that the ancestral forms of *Lolium* have been most like the present perennial ryegrass (Bulińska-Radomska and Lester 1988). An evolutionary trend in reduction of life-cycle from the perennial forms to the annual taxa seems to be supported by the combined analyses of ITS and *trnL*-F sequences (Catalan et al. 2004). Alternatively, most authors agree that the common ancestor has its closest affinity with *L. rigidum* (Malik 1967; Charmet and Balfourier 1994). But somewhat an intriguing calculation when the differentiation of the genus into species has begun was proposed. Firstly, using isozymes Charmet and Balfourier (1994) postulated that the evolution of *Lolium* has started about 10 000 years ago in close association with primitive agriculture. When the data from chloroplast DNA were employed it turned out that differentiation of the genus into species has begun much earlier, about one million years ago in the Middle East, at the same time as reported for the genus *Triticum* (Charmet et al. 1997). So, there is tremendous discrepancy.

The uncertainties related with *Lolium* phylogeny contribute greatly to the ambiguity of the taxonomic classification of the genus. Moreover the frequent revision of classification schemes using the different names for slightly dissimilar variants of the same species makes them confusing. Terrel (1968), for example, found almost 500 published names for the dif-

ferent species, and many of them were synonymous. At least 30 different synonyms can be found in the Integrated Taxonomic Information System of the USA (2007) and Flora Europea (2007). The fewest synonyms have been recorded for self-pollinated species *L. persicum* and *L. remotum* while the most have been noticed for *L. multiflorum* and *L. loliaceum* (Table 1.2). The latter species was classified by Terrel (1968) as *L. rigidum* var. *rottbollioides*, Malik (1967) referred to it as an intermediate species between *L. rigidum* and *L. temulentum* while some authors used *L. subulatum* as a synonym of *L. loliaceum* (Bennett 1997). The taxonomy of ryegrasses is additionally complicated by different mode of classification in the USA and Europe.

Table 1.2. Accepted and synonymous names for *Lolium*

Accepted names	Synonyms	Common names	Mode of reproduction
***Lolium canariense* Steud**			intermediate
***Lolium loliaceum* Bory &Chaub**	•*L. crassiculme* Rech.f. •*L. lepturoides* Boiss. •*L. rigidum var. rottboliodes* Heldr. ex Boiss •*L. rigidum* ssp. *lepturoides* (Boiss.) Sennen & Mauricio •*L. subulatum* Vis •*Rottboellia loliaceum* Bory & Chaub		autogamous
***Lolium persicum* Boiss & Hohen. ex Boiss.**	•*L. dorei* Boivin •*L. dorei* var. *laeve* Boivin	•Persian darnel •Persian ryegrass	
***Lolium remotum* Schrank**	•*L. temulentum* ssp. *remotum* (Schrank) A.Löve & D. Löve •*L. linicola* A.Braun	•Flax darnel	
***Lolium temulentum* L.**	•*L. arvense* With. •*L. temulentum* var. *arvense* (With.) Lilja •*L. temulentum* var. *leptochaeton* A. Braun •*L. temulentum* var. *macrochaeton* A. Braun	•Darnel •Darnel ryegrass •Poison darnel	
***Lolium multiflorum* Lam.**	•*L. aristatum* Lag. •*L. gaudinii* Parl. •*L. italicum* A. Braun •*L. multiflorum* var. *diminutum* Mutuel •*L. multiflorum* var. *muticum* DC. •*L. perenne ssp. multiflorum* (Lam.) Husnot •*L. perenne* var. *aristatum* Willd. •*L. perenne* var. *multiflorum* (Lam.) Parnell •*L. siculum* Parl.	•Annual ryegrass •Italian ryegrass •Darnel	allogamous
***Lolium perenne* L.**	•*L. cristatum* L. ex Nyman •*L. multiflorum* var. *ramosum* Guss. ex Arcang. •*L. perenne* var. *cristatum* Pers. ex B.D. Jackson •*L. perenne* ssp. *perenne* L.	•Crested ryegrass •English ryegrass •Perennial ryegrass	
***Lolium rigidum* Gaudin**	•*L. parabolicae* Sennen ex Samp.•*L. perenne* ssp. *rigidum* (Gaudin) A.& D. Löve •*L. strictum* C. Presl	•Stiff ryegrass •Wimmera ryegrass	
Hybrid species			
Accepted name	**Hybrid between:**		
Lolium x hybridum	•*L. multiflorum x L. perenne*		
Lolium multiflorum x remotum	•*L. multiflorum x L. remotum*		
Lolium multiflorum x temulentum	•*L. multiflorum x L. temulentum*		
Lolium perenne x remotum	•*L. multiflorum x L. remotum*		
Lolium perenne x rigidum	•*L. perenne x L. rigidum*		

Summarized on Flora Europea (2007) and the Integrated Taxonomic Information System (2007)

In the absence of fossil records ryegrasses are classified according to easily detected characteristics that, hopefully, do not alter much with environment. More than twenty characters related with spike and leaf morphology are usually taken into account (Table 1.3). Each classification considers different characters as the most important ones. The spikelet number, length of the glumes, shape of lemma, length of caryopsis, presence or absence of awns and longevity are given prominence in most classification schemes. But morphology alone failed to unambiguously resolve systematic relationships because some degree of

overlap between species has been observed (Bennet 1997; Loos 1993a; Bennet et al. 2000). To further complicate the picture all allogamous species can intercross freely and produce a spectrum of intermediate forms. No completely negative results are obtained even when out-pollinated types are intercrossed with self-pollinated species (Jenkin 1959). Therefore, Terrel (1968) recommended classifying only the extremes as species and also recognising introgressions between them. As a result, it has been a habit to classify intermediate forms as hybrids and dignifying them with a specific "Latin" binomial name. At the moment at least five interspecific hybrids are recorded in Flora Europea (2007) however, they are not accepted in the Integrated Taxonomic Information System of the USA (2007).

In general, the genus can readily be divided into two sections according to whether the plants are normally self-pollinated or out-pollinated. The allogamous forms are supposed to be evolutionary younger than autogamous ones. This segregation is consistently observed in morphological (Jenkin 1959; Loos 1993a), isozyme (Loos 1993b; Charmet and Balfourier 1994; Bennett et al. 2002), rDNA (Charmet et al. 1997; Warpeha et al. 1998) and low-copy nuclear sequence studies (Polok 2005). On the other hand, in some studies inbreeding *L. remotum* can also be clustered with out-breeding *L. multiflorum* (Bennett 1997) or *L. rigidum* (Bulińska-Radomska and Lester 1985). There is no strong consensus on the phylogenetic placement of *L. loliaceum* (*L. subulatum*), probably because this species has been rarely included in analyses.

Further distinction within these two groups is even more difficult. Terrel (1968) recognized the inbreeding species as one group except of *L. subulatum* (*L. loliaceum*) but the majority studies separate them easily (Loos 1993a; b; Charmet Balfourier 1994). In contrary, little morphological and biochemical differentiation is found between cross-pollinated species (Terrel 1968; Loos 1993 a; b). They show very wide intraspecific variation and stable types exist, which do not easily fall into any of three outbreeders. Numerous natural hybrids are also observed. Consequently Terrel (1968) classified the allogamous species as one group with *L. rigidum* being a polymorphic complex made up of several elements. More recently, Bulińska-Radomska and Lester (1985) postulated the re-classification of *L. multiflorum*, *L. perenne* and *L. rigidum* into three subspecies. But the above conclusion seems implausible to the advocates of traditional "morphological view". Explaining the varying degrees of similarity between out-breeding species by the recent evolutionary divergence Stammers et al. (1995) tried to find agreement between molecular data and classical taxonomy.

It is a pity that most conclusions regarding *Lolium* phylogeny are based on morphology or a limited number of isozyme loci. Even though this classical approach has been complemented by DNA analysis, nearly all studies employed only the data from chloroplast genome (RFLP of *trn*, *rbcL*, *psbC* fragments: Charmet et al. 1997; RFLP of *trn*, *psb* fragments: Balfourier et al. 2000; *trnL-F* sequences: Catalan et al. 2004). They support for generic relationships between *Lolium* and *Festuca*, but support for relationships among *Lolium* is minimal. In the consensus tree interfered from the *trnL-F* sequences all representatives of this taxon collapsed in a large polytomy with representatives of the *F. pratensis* complex, *F. fontqueri*, *F. gigantea* and *Micropyropsis* (Catalan et al. 2004). It should also be kept in mind that evolutionary history based on cpDNA is that of the maternal lineage of the species. A comparable comprehensive nuclear data set from the genus *Lolium* is lacking. So far published studies have incorporated mainly the internal transcribed spacer (Charmet et al. 1997; Gaut

Table 1.3. Taxonomic characters of *Lolium* species

Species	*L. canariense*	*L. loliaceum*	*L. persicum*	*L. remotum*	*L. temulentum*	*L. multiflorum*	*L. perenne*	*L. rigidum*
Habit	Annual	Annual	Annual	Annual	Annual	Annual/biennial	Perennial	Annual
Plant colour			Bright green, shiny	Yellowish green	Bluish grey green	Vividly green	Vividly green	Shiny, vividly green
Growth habit	Erect	Erect	Erect	Erect	Erect	Erect	Erect	Erect/Prostate
Culm height	30-67 cm	9-52 cm	14-45 cm	40-80 cm	20-120 cm	20-100 cm	12-80 cm	26-77 cm
Spike length	10-20 cm	4-23 cm	3-21 cm	2-23 cm	5-40 cm	15-45 cm	3-30 cm	3-30 cm
Rhachis	Semiterete	Angular	Semiterete	Semiterete	Semiterete	Semiterete	Semiterete	Subcylindrical
Spikelets	Oblong, laterally compresse	Cuneate, laterally compressed	Oblong, laterally compressed	Oblong, laterally compressed	Oblong, laterally compressed	Oblong, laterally compressed	Oblong, laterally compressed	Oblong, laterally compressed
N° of spikelets on a spike	7-15	4-21	3-12	3-20	5-26	5-38	5-37	2-20
Spikelet width	1-4 mm	1-2 mm	1-7 mm	1-5 mm	3-8 mm	3-5 mm	2-4 mm	1-4 mm
Spikelet length	6-24 mm	3-7 mm	12-22 mm	5-16 mm	12-26 mm			
N° of florets on a spikelet	5-9	2-5	4-7	2-10	2-15	11-22	5-11	2-9
Awns	+	+/-	+	-	+/-	+	-	-
Glume	Persistent	Lateral, persistent,	Persistent	Persistent	Persistent	Persistent	Persistent	Persistent
Glume length	3-28 mm	5-27 mm	7-23 mm	5-16 mm	7-30 mm	5-18 mm	4-15 mm	4-20 mm
Lemma	Lanceolate, 5-7 -veined	Elliptic, 5-veined	Lanceolate, 3-5 -veined	Ovate, 3-7 -veined	Elliptic to ovate, 5-9 -veined	Oblong, 5-veined	Oblong, 5-veined	Lanceolate, 3-5 -veined
Lemma surface	Glabrous	Smooth or scabrous	-					
Lemma length	3-10 mm	5-6 mm	7-12 mm	3-5 mm	4-9 mm	4-8 mm	4-9 mm	3-8 mm
Palea	1 times length of lemma	As long as the lemmas	Longer than the lemmas	As long as the lemmas	As long as the lemmas	Shorter than the lemmas	Shorter than the lemmas	Shorter than the lemmas
Leaf vernation		Rolled		Rolled	Rolled	Rolled	Folded	Rolled
Leaf sheath	Glabrous, loose	Glabrous, inflated,	Glabrous, closed	Glabrous	Glabrous, open	Glabrous, open	Glabrous, closed	Glabrous, swollen
Leaf blade length	5-21 cm	6-12 cm	3-20 cm	10-27 cm	13-28 cm	6-30 cm	5-30 cm	10-17 cm
Leaf margins	Rough	Smooth	Rough	Rough	Smooth	Rough	Slightly rough	Smooth
Leaf surface	Smooth or scaberulous	Smooth with a midrib prominent	Rough	Smooth	Smooth or with trichomes	Smooth with a midrib prominent	Smooth	Smooth
Collar		Glabrous			Glabrous	Narrow, yellowish	Glabrous	
Auricles	Falcate	Glabrous	Present only on later	Absent or falcate	Falcate, glabrous	Falcate, glabrous, narrow	Falcate, glabrous	Absent or falcate
Ligule	Membranous, 1-2 mm	Membranous, 1-2 mm	Membranous 1-2 mm	Membranous, 1-2 mm	Membranous, 1-3 mm	Membranous, 1-2 mm	Membranous, 1 mm	Membranous, 1-2 mm
Caryopsis	With adherent pericarp, ellipsoid. 3-5 mm long	With adherent pericarp, ellipsoid,. 1-5 mm long	With adherent pericarp, linear, 5-7 mm long	With adherent pericarp, oblong 5-7 mm long				With adherent pericarp, fusiform 2-6 mm long
Roots					Fibrous	Branched, fibrous	Branched with adventitious roots	

Summarized on the basis of Terrel 1968; Szafer et al. 1986; Weiller et al. 1995; Bennet 1997; Bussan and Trainor 2001; Clayton et al. 2006

et al. 2000; Catalan et al. 2004) while both low copy nuclear genes and genome scanning molecular markers are avoided. Although the tree resolution based on ITS was better than this based on cpDNA, its interior nodes were consequently not resolved with certainty. Autogamous *L. temulentum* formed a single cluster with allogamous species, *L. multiflorum* and *L. perenne*, while *L. rigidum* was grouped with *L. remotum* and *L. subulatum* (*L. loliaceum*). Therefore, Charmet et al. (1997) concluded that the genus *Lolium* should be regarded as a single entity as far as its uses as a genetic resource for the breeding programmes of cultivated species are concerned.

The knowledge about evolutionary history of the genus *Lolium* has both theoretical and practical implications. Firstly, the phylogenetic relationships can serve as the basis for a taxonomic system. Secondly, the ryegrasses are also crop plants and breeders wish to know whether a particular plant is of the desired cultivar. Therefore, the especially important is the determination of biological status of *L. multiflorum* and *L. perenne*, the most important forage grasses over the world. Meanwhile these both species have become a subject of the greatest dispute.

1.5. TAXONOMIC STATUS OF *L. MULTIFLORUM* AND *L. PERENNE*

The point at issue is whether *L. multiflorum* and *L. perenne* can be regarded as distinct biological species or not. Indeed, in the most of European taxonomic systems, including Flora Europea (2007), both ryegrasses hold the status of taxonomic species. They are distinguished on the basis of several morphological characters from which the growth habit, leaf vernation and presence or absence of awns are the most discriminant (Table 1.3). But even these characters are not absolute and a morphological feature of one species is also observed in another. *L. perenne*, perennial ryegrass, is the only long-lived perennial in the genus while *L. multiflorum* (annual ryegrass, Italian ryegrass) is rather an annual species. However, it is not a true annual and may behave as a biennial or even short-lived perennial depending on environmental conditions (Cosgrove et al. 1999). The annual cultivated type of *L. multiflorum* may be only a segregate from the non-annual Italian ryegrass (Jenkin 1959). The perennation is encoded by a few genes and the gradation from annual through winter annual to perennial forms is observed in many plant species, for example in cultivated barleys (Briggs 1978).

The attempts to properly identify *L. multiflorum* and *L. perenne* using the dichotomous key can be very frustrating due to the overlap of variation in the majority of taxonomic characters (Table 1.3). This lack of hiatus, that is a typical feature of biological species, is so confusing that it is neither discussed nor investigated further. Bennett (1997) and Bennet et al. (2000), for example, concluded that *L. multiflorum* and *L. perenne* showed a clear distinction despite the fact that their populations partially overlapped on the principal component scatterplot. Similarly, Loos (1993a) stated that both species were distinct though all populations of Italian and perennial ryegrasses were similar and the first principal component did not separate them. The advocates of *L. multiflorum* and *L. perenne* separation point out that the overlapping range of variation indicates independent evolutionary processes that have led to many different forms in each species. These forms can be classified as varieties while

the intermediate forms that do not easily fall into one of the species are regarded as a result of limited introgression. For example, the introgression was observed at some enzymatic loci. Even so, these introgressions were not sufficient to hide the clear-cut divergence between the two species (Charmet and Balfourier 1994; Bennet et al. 2002). Although the dendrograms seem do not support this view because *L. multiflorum* and *L. perenne* populations were always mixed altogether.

On the other hand the difficulties in separation of *L. multiflorum* and *L. perenne* on the basis of morphology and more recently on enzymatic and DNA level questioning their species status. The searches for a marker that would be able to distinguish both ryegrasses unambiguously is a never ending story and so far without a significant success. The seedling root fluorescence also known as the Gentner's test has been widespread as a supplement to morphological data since it discovery in 1929. Generally, seedling roots of annual ryegrass fluoresce when placed under ultraviolet light, and those of perennial ryegrass do not fluoresce. But the test is not exact and both non-fluorescent *L. multiflorum* cultivars and fluorescent *L. perenne* exist. Several attempts have been made to improve the identification of ryegrasses by means of isozymes (Hayward and MacAdam 1977; Nakamura 1979; Greneche et al. 1991) and DNA (Baker and Warnke 1999). The former method is generally based on differences of band intensity and therefore, prone to human judgment errors. The latter employs the linkage between DNA marker and the gene controlling the taxonomic character such as first year flowering. Unfortunately, the linkage with first year flowering may not be the best genetic separation to use because there are some perennial plants flowered without vernalization in the first year of cultivation. Thus, the DNA test suffers the same problems as the morphological separation or root fluorescence. The behavior of *L. multiflorum* and *L. perenne* is much the same to the different cultivars or ecotypes where such difficulties in finding unique markers are common. Wheat cultivars were indistinguishable even when AFLP (Amplified Fragment Length Polymorphism) was used. It was only when high variable transposon sequences were employed; it was possible to find cultivar specific markers (K. Polok, unpublished data). Similarly, botanical varieties of diploid bristle oat, *Avena strigosa* var. *glabrescens* and *A. strigosa* var. *subpilosa* did not differ on the DNA level (Zielinski and Polok 2005).

The absence of a strong species boundary between *L. multiflorum* and *L. perenne* can be both a curse and a blessing. It is a curse on taxonomists because it is difficult to justify the separation of species that can interbreed freely and they continue to do so indefinitely in later generations. That is probably why the adherents of species status of *L. multiflorum* and *L. perenne* explain that their hybridization is a recent phenomenon, not observed or limited in the past. At present these species are sown beyond their natural distribution and therefore, they come into contact. This resulted in frequent hybridization and gene flow (Bennett et al. 2002). But the full crossability of *L. multiflorum* and *L. perenne* is also a blessing because it can be the strongest evidence supporting the hypothesis that they are not biological species. Naylor (1960) was the first who proposed to classify Italian and perennial ryegrass as one species. More recently the same suggestion was made by Bulińska-Radomska and Lester (1985) based on morphology and isozymes and Zielinski et al. (1997) based on isoenzymes and RAPD (Random Amplified Polymorphic DNA).

According to the biological species concept, species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups (Mayr 1996). Under this definition species are biological and evolutionary entities and therefore, it is perhaps less arbitrary definition than the other species concepts. The speciation is gradual process that in many cases yields intermediate outcomes. It is a challenge to distinguish at what particular points of evolutionary time two populations are if the hybrids are partially fertile (Avise 2004). But two situations are black-and-white - the presence of a full reproductive barrier that is typical of good biological species and its lack - the feature of conspecific populations. The hybridization between closely related but still biological species always leads to the reduction of fertility and viability of F_1. The hybrids between closely related *Hordeum vulgare* and *H. bulbosum* are not developing because the chromosomes of *H. bulbosum* are rejected during first days after pollination. Despite *L. perenne* can be successfully intercrossed with *F. pratensis* (2n=14), *F. arundinacea* (2n=42) and *F. gigantea* (2n=42) and all F_1 hybrids reach the flowering stage, they can be propagated only vegetatively. These F_1 hybrids are often functionally male sterile and female fertility is also very low. The similarly poor results and no ovary stimulation are observed when *L. multiflorum* or *L. perenne* are crossed with *L. remotum* or *L. temulentum* (Jenkin 1959).

On the other hand, the absence of reproductive barriers raises no doubts that we are dealing with conspecific populations irrespective how they are classified by plant taxonomists. This is probably the situation we observe in *L. multiflorum* and *L. perenne*. The F_1 hybrids always reached the full maturity, were fully fertile and seedlings were established without any problems (Jenkin 1959). What's more the heterosis effect i.e., higher vigour than in parents has been frequently observed (Polok 2005). Another issue is whether both species can intercross in nature or they are separated geographically. Data from the fluorescence test suggested the uncommon hybridization in nature because two species have hardly overlapping area (Arcioni and Mariotti 1983). Such conclusions are untenable in view of data from Western Australia. The hybridization has occurred to such extent that few populations can now be recognised as distinct species (Bennett et al. 2002). Similarly, the entire European population is a group of hybrids with continuous variation (Cresswell et al. 2001). The argument about recent origin of hybridization also seems not plausible. Although, because long lasting cultivation of *L. multiflorum* and *L. perenne* their natural range has not been known, and we can speculate that their speciation may have occur allopatrically followed by secondary range overlap, the primary sympatric distribution of both species seems more probable. As noted by Jenkin (1959), *L. perenne* is rather a more southerly type originated from the Mediterranean region or western Asia and it has migrated northwards along the coastal regions of Western Europe as a camp-follower of man. The types were found in North Africa which are adapted to very extreme southerly conditions. One difficulty in the above hypothesis involves the lack of comprehensive experimental data dealing with crossability of *L. multiflorum* and *L. perenne* except of old work of Jenkin (1959). Nevertheless, the difficulties in sustaining the stability of strains if both species are sown within pollination distance and the intermediate phenotype of *Lolium* x *hybridum* are believed to be associated with the lack of reproductive barriers. This argument has taken precedence of the other in taxonomic considerations in the USA. Thus, the Integrated Taxonomic Information System of the USA (2007) only accepts *L. multiflorum* and *L. perenne* as subspecies within *L. perenne*.

Reproductive isolating barriers develop as a by-product of genomic divergence. The speciation process is associated with the conversion of genetic variability within a species to between-species genetic differences. Together with the divergence of populations, the similarity of their gene pools is falling down. The universal for the whole plant and animal kingdom, genetic similarity (or gene identity) index is a good measure of population diversity allowing multiple comparisons between many populations and species. Although, several methods of the genetic identity estimation have been developed, the overall idea is the same - the genetic identity is very low, close to zero, in well-defined biological species with the full reproductive barrier while it is close to one in populations of a species. Between these extremes is a huge gap, informing about the stage of divergence (Zielinski and Polok 2005). The data from the allozyme era documented very well a wide spectrum of these evolutionary processes in plants finally leading to revision of our knowledge about speciation. Unfortunately, such analyses in *Lolium* were focused only on cultivar differentiation. The early enzymatic comparisons of *L. multiflorum* and *L. perenne* that proved to exhibit the allozyme divergence more connected with populations than species were quite confusing (Loos 1993b; Charmet and Balfourier 1994). Alongside the advances in molecular methods the speciation process can be followed much more precisely at various stages. And again, the first empirical data were clear: *L. multiflorum* and *L. perenne* are almost indistinguishable based on both chloroplast and nuclear high-copy DNA (Charmet et al. 1997). Especially, the 100% identity of ITS sequences isolated from individuals of both species has been surprising (Gaut et al. 2000). However, the caution should be made about data from a single ITS sequence or any other single-gene comparisons. Otherwise, genome scanning molecular markers permit endless opportunities in speciation studies. Their tremendous phylogenetic power resides in the extraordinary amount of cumulative information from vast numbers of loci. Even though these high-throughout technologies have been developed in ryegrasses, they have been used primarily for breeding purposes and hardly any studies are dealt with the comparison of *L. multiflorum* and *L. perenne*. Finally, some data are coming from larger phylogenetic studies of *Lolium-Festuca* complex. The pioneering study of Stammers et al. (1995) and Zielinski et al. (1997) based on RAPD markers indicated the very low divergence between Italian and perennial ryegrass but they included only cultivars and thus, could be biased by breeding activities. Other noteworthy analyses including both cultivars and ecotypes showed very high genetic identity (i.e., I = 0.96) estimated from RAPDs and ISJs (Intron Splice Junction polymorphism), well within the range of values normally associated with conspecific populations (Polok 2005). Strangely, the discovery of high genetic identity did not clinch the case for species status of *L. multiflorum* and *L. perenne*. Instead, it prompted development of alternative explanations that assume a recent origin of hybridization or extensive introgression in disturbed habitats. Indeed, one aspect of these early data must be stressed. The phylogenetic conclusions interfered from a single gene, or even single method can reflect rather the evolution of a gene or sequences revealed by a given method. Only comprehensive analyses taking into account all levels of genetic differentiation (morphological, enzymatic and molecular) as well as employing different technologies are able to resolve reliably the phylogenetic history of a species. The genus *Lolium* is still pending for such multidimensional approaches and up to then the status of two the most important forage grasses seem to be insoluble.

1.6. APPLICATION OF MOLECULAR DATA IN EVOLUTIONARY GENETICS

The effort to group species within plant taxa was traditionally based on the morphology of characteristic structures. But morphology alone failed to unambiguously resolve systematic relationships. The advantage of all DNA methods is that they provide direct access to the genomes in many organisms and thus, they have impact on our theoretical insight about evolution. Alongside with the developing of PCR technologies we get access to practically unlimited number of markers that permit to investigate the phenomena that were previously inaccessible to evolutionary genetics.

Although a diverse array of molecular approaches is now available for elucidating relationships and evolutionary processes, the phylogenetic analysis has relied primarily on the chloroplast genome. Most studies have employed restriction site analysis, sequencing or PCR analysis of several single copy regions i.e., *rbcL* that encodes the large subunit of ribulose1,5-bisphospate carboxylase/oxygenase (RUBISCO), *atpB* - encoding the -subunit of ATP synthase, *ndhF* - encoding a subunit of chloroplast NADH dehydrogenase, *matK* - encoding a maturase involved in the splicing type II introns, *rpoC2* - encoding - subunit of RNA polymerase, and ribosomal protein *rps4*. Other sequences of the chloroplast genome also have phylogenetic potential, including the *atp-rbcL* intergenic region, *trnL* intron, intergenic spacer between the *trnL* 3'exon and the *trnF* gene (Soltis et al. 2000; Avise 2004). The advantage is that the chloroplast genome is small and thus, relatively easy to examine. As a result a wide range of applications exists from the level of species, through genus and family to higher taxa. For example, the sequence of the RUBISCO indicated that the closest relative of the grasses is the genus *Joinvillea*, the only member of the family Joinvilleaceae (Kellog 1998). Also, before molecular analyses, the bambusoids were considered early diverging grasses. However, particularly the data from the *rbcL* and *ndhF* genes proved that the anomochlooids represent the earliest diverged lineage while the bambusoids fall within a monophyletic BEP clade (Gaut 2002). A cautionary point should be made about phylogeny based on chloroplast genes. The cpDNA tree resolution depends on the "evolutionary age'' of the group of interest and it fails to resolve the topology at the genus and species level for the recent lineages. This was demonstrated for the Triticeae tribe (Catalan et al. 1997) as well as in the complex *Festuca-Lolium* (Charmet et al. 1997; Balfourier et al. 2000). The low resolution of cpDNA trees at lower taxonomic level results from slow evolution of cpDNA in terms of both primary nucleotide sequence and gene rearrangement (Avise 2004). The other disadvantage involves the ancient chloroplast capture via introgressive hybridization that will bias estimates of phylogeny. Given the apparently high frequency of cytoplasmic introgression in plants, the phenomenon is among the most common causes of phylogenetic disorders (Soltis et al. 2000).

Despite the large size of the nuclear genome and huge diversity of genes, only several sequences have been used in phylogenetic analyses. The special emphasis has been given either to high-copy ribosomal loci in nuclear DNA (rDNA) or spacer regions. The rDNA includes the tandem repeat structure, which makes it easy to amplify, clone and sequence. These genes are also found in all organisms. Both small subunit (18S) and large subunit (26S) rDNA sequences are highly conserved. They have been useful primarily to access a variety of higher-level phylogenic questions, whereas the 5.8S rDNA has rarely been used.

The internal transcribed spacer (ITS) is commonly used due to easy amplification and sequencing using universal primers as well as the near uniformity of paralogous. The main reasons for this homogeneity, typically known as concerted evolution, include unequal crossing-over, gene conversion, transposition and slippage (Soltis et al. 2000). Rapidly evolving ITS sequences are thought to be the best suited for comparing species or closely related genera. For example, the ITS data suggest that currently recognised sections within *Aegilops* should be reconsidered (Wang et al. 2000). But the utility of ITS region in broader study of grasses may be limited. Despite the fact that ITS sequences have enabled to resolve boundaries of families and subfamilies within Poaceae, the support for relationships among most tribes is minimal and the sequences are difficult to align (Hsiao et al. 1999). The lack of phylogenetic resolution is also demonstrated within the genus *Lolium* (Charmet et al. 1997; Gaut et al. 2000). These works indicate that the evolution of ITS regions may be more complex than thought initially, and also suggest the caution in phylogeny reconstruction at least in grasses. The rapid concerted evolution of intergenic spacer sequences between nuclear tRNA genes organized in tandem arrays also permits their use for inferring phylogeny. The utility of the spacer between leucine tRNA genes was demonstrated for the liverworts from the genus *Pellia* (Fiedorow et al. 2001) but it failed to differentiate *L. multiflorum* and *L. perenne* (Polok et al. 1997).

The low copy nuclear genes are often avoided in evolutionary studies because identification of strictly orthologous sequences may be confounded by the presence of multiple related loci in the genome. A simple way to sample putatively orthologous loci is to use locus-specific amplification primers. Most of the studied nuclear genes are members of small multigene families i.e., the phytochrome gene family (PHY), the glutamine synthetase gene family (GS), glucose-6-phospate isomerase genes *(Gpi),* alcohol dehydrogenase genes *(adh)*, vicilline genes, b-amylase genes, and MADS-box genes (Soltis et al. 2000; Mathews et al. 2000; Mason-Gamer 2005). Putatively single-copy genes have been examined less frequently and they include the nuclear gene for chloroplastic glyceraldehyde 3-phosphate dehydrogenase (*gap3*), granule-bound starch synthase gene (*waxy*) and acetyl-CoA caboxylase gene (*Acc*). So far, phylogenetic data from throughout Poaceae, sampling different taxonomic level are available only on *waxy* gene (Mason-Gamer et al. 1998) and phytochrome B (Mathews et al. 2000). They all indicate that the family is a single lineage. Eventually, the utility of the nuclear genes encoding alcohol dehydrogenase (Gaut and Clegg 1991), acetyl-CoA carboxylase (Huang et al. 2002) and b-amylase (Mason-Gamer 2005) were demonstrated on a few members of the grass family with special emphasis on Triticeae. In general, the revealed relationships were consistent with the known facts but the gene trees have not completely clarified the complexity of the family. The genus *Lolium* was represented only in two of these studies. A single *L. perenne* ecotype probably collected by the authors was included in the analysis of phytochrome B (Mathews et al. 2000) and two *L. rigidum* accessions from Australia - in the analysis of acetyl-CoA carboxylase (Huang et al. 2002). Additionally, the utility of acetyl-CoA carboxylase in *L. multiflorum* and *L. perenne* was studied by Polok et al. (1997). Obviously, there is lack of comprehensive data enabling to estimate the utility of the above sequences in the phylogenetic analysis of the genus *Lolium*. Although, there is a clear picture that the tree resolutions for the majority of low-copy sequences are too low to accurately reflect pattern

of evolution within the recently diverged grass genera. Moreover each phylogenetic analysis based on single sequences should be treated as provisional.

Even closely related taxonomic species normally differ in many genetic features, not just one or a few. If two species differ in only 1% of genes, that is rather a small genetic distance, and if their genomes contain roughly 20000-30000 genes, as it can be estimated from the average gene density in grasses (Feuillet and Keller 2002) and genome size, then a total of about 200-300 genes are different. What is more, total genetic diversity that could arise from non-coding, repetitive sequences, contributing to as much as 70-80% of grass nuclear DNA, vastly exceeds that from the unique genes. Even a small fraction of these genetic changes provides potential molecular markers for analyzing of speciation. Protein electrophoresis was one of the earliest methods disclosing this genetic variation and provided a huge amount of data about the mechanism of microevolution. It still remains a simple, popular and powerful method for generating Mendelian nuclear markers in many evolutionary applications. In fact, all data about phylogenetic relationships within the genus *Lolium* have been mainly based on enzyme electrophoresis (Loos 1993b; Charmet and Balfourier 1994; Bennet et al. 2002). It is also a standard method in analyzing cryptic (sibling) species i.e., morphologically similar species between which the reproductive barrier exists (Avise 2004). Such species consistently exhibit the different enzyme variants that were excellently shown in many animal, fungi and plant species including liverworts (Zielinski 1987; Polok et al. 2005b; Bączkiewicz et al. 2007).

Invention of the polymerase chain reaction (PCR) led to the revolution in the acquisition of new genetic markers. The enormous attraction of these methods is the possibility to amplify a number of discrete DNA products both from gene-rich and gene-poor regions, the latter primarily consisted of repetitive sequences. The genome scanning molecular markers have a wide variety of applications along the phylogenetic hierarchy, especially in estimating pairwise similarity (distance) between individuals from the enormous number of loci. Of course, each molecular technique can be prone to a bias resulted from the different nature of polymorphism generated. For example, primers complementary to known sequences at exon-intron junctions (ISJ) preferably amplify the low-copy sequences. Perhaps this was the reason that the method was especially powerful in finding the markers correlated with mutant phenotypes in pea, *Pisum sativum*, barley, *Hordeum vulgare* (K. Polok, unpublished data), the markers correlated with the unique crown and stem type in Scots pine, *Pinus sylvestris* (Polok et al. 2005a) and the separation of cryptic species of a liverwort *Pellia endiviifolia* (Polok et al. 2005b). Another approach to generate low-copy markers takes advantage of the fact that some PCR primers successfully amplify not only target DNA from the species from which they were derived, but also homologous DNA across broader taxonomic groups. As it has been demonstrated by Zielinski and Polok (2005) such sets of primers can be easily developed using conservative bacterial genes whose descendants can be found in higher plants. One excellent example is bacterial KatG gene encoding catalase-peroxidase in *Mycobacterium tuberculosis* that is commonly used for strain identification. Taking into account that all plant and animal catalases and peroxidases are derived from a common prokaryotic gene sequence it could be assumed that primers complementary to *KatG* gene at least partially can amplify homologous sequences in plants. The method later on referred as katG based polymorphism (katG) have proved to be superb for identify-

ing species at different taxonomic level (Nowak and Polok 2005; Szczecińska et al. 2006; Bączkiewicz et al. 2007). The system was also successful in differentiation of allogamous *L. temulentum* from autogamous *L. multiflorum* and *L. perenne* but the later two species were indistinguishable (Polok 2005). Similarly, primers of wide taxonomic latitude can be designed on the basis of flanking sequences of *M. tuberculosis* insertion element, IS6110. Well-documented examples of species identification using IS6110 primers are in grasses, liverworts from the genus *Pellia*, mosses from the genus *Sphagnum*, *Acutifolia* section and closely related *Pinus sylvestris* and *P. mugo* (Zielinski and Polok 2005).

On the other hand, there is a great choice between the methods revealing polymorphism of moderate and high copy repetitive sequences. The best known are microsatellites (SSR - Simple Sequence Repeats) that amplify the short tandem repeats and their applications are primarily related with identification of individuals for forensic or breeding purposes. On the positive side, the highly mutable nature of SSR loci increases the possibility of having many allelic variants at each locus but alleles identical in size are not necessarily identical by descent. On the negative side, this characteristic of microsatellites can often cause serious interpretative difficulties (Avise 2004). Additionally, the laborious primer development for each species makes this method unpractical for phylogenetic analyses although it can be sometimes applied in closely related taxa as long as the cross-species amplification can be obtained. For example, about 80% of SSR loci isolated from genomic library of *L. perenne* were also identified in *L. rigidum* and 71% in *L. multiflorum*. Amplification was slightly lower in the *Festuca* species (Jones et al. 2001). Efficient cross-species amplification was also reported for inbreeding *Lolium* taxa (Kubik et al. 1999).

The most advanced technologies such as Amplified Fragment Length Polymorphism (AFLP) can generate up to 50-100 bands in a single PCR reaction without prior sequence knowledge. This means that thousands of bands can be revealed in a relatively short time period to scan a larger portion of genomes in any number of taxa than ever before. Studies employing AFLP have been highly fruitful at the level of conspecific populations, also in perennial (Guthridge et al. 2001; Roldan-Ruiz 2001) and Italian ryegrass (Cresswell et al. 2001) but promising results are also obtained at the generic level. The tree resolution based on AFLP profiles differentiated most of seven *Lolium* species with the only exception of *L. multiflorum* and *L. perenne* (Polok et al. 2006). In principal, the AFLP technique was developed to amplify the restriction site polymorphism however, it has been known that an AFLP gel may have a mixture of restriction site, microsatellite and transposon polymorphism. Hence, a variety of modifications can be done to reveal only one kind of polymorphism. For example, in SSAP (Sequence Specific Amplification Polymorphism) approach, the primers are constructed to amplify the polymorphism resulted from insertion sites of transposable elements. The trick is to design PCR primers specific to the unique flanking regions of a given transposon (Kumar et al. 1997). Molecular markers based on transposons have proved to be more informative than non-transposon based marker methods. Some of these, which have been transposing in the recent past, are extremely polymorphic within species and may be used in linkage analysis or intra-specific genetic diversity studies. Other transposons which were active several million years ago should be more useful in elucidating phylogenetic relationships between species (Pearce et al. 1999). By combining data from several transposons, a clear picture can be obtained of complex species groups. Although transposable elements

are continuously entering new sites, mutations caused by their insertions are probably much less frequent than are point mutations in most organisms. Over millions of years of evolution, transposons have achieved a balance between detrimental effects of mobilization and long-term beneficial effects on a species through genome modifications. As a result, about 90% of transposons remain reasonable stable (Kazazian 2004) but they can be reactivated by stress or other unusual events. This can be the beginning of population differentiation leading to speciation. Therefore, transposons are especially useful in analysis of recently diverged, closely related taxa. In recent years transposons have been shown to be present throughout the plant kingdom. They have played a significant role in the evolution of grasses being responsible for genome size variation. Despite transposons play important role in speciation of Poaceae they have never been exploited in resolving phylogenetic relationships within the genus *Lolium*.

Closely related taxa often tend to be more similar in phenotype than distant taxa. Whenever a phylogeny is known with reasonable assurance, morphological, behavioral, or other organismal features may be alternative states of composite attributes (such as awns, perennation in *Lolium*). An implication of this is that evolution of higher taxa might have been driven by rare mutational changes in single major genes (Avise 2004). On opposite, closely related taxa usually differ in discrete traits whose underlying model of inheritance is multifactorial and polygenic. Selection would favor new multigenic combinations that would create a discrete shift in morphology. This model assumes that evolution of closely related taxa is depended on multiple genes, each with a small effect on phenotype - i.e., quantitative trait loci (QTL). The use of DNA makers in conjunction with experimental crosses enables to map genomic position of these “speciation QTLs”. The approach is virtually the same as QTL mapping for breeding purposes and involves the crosses between two species in question. An argument against this methodology is that it can be applied only if both interspecific hybrids and their offspring can be produced. Second, such approach needs a genetic map of at least modest resolution. Nevertheless, the fruitful results were obtained in maize, in which 22 QTLs affecting the seven traits responsible for the evolution of maize from teosinte were identified (Lauter and Doebley 2002). This strategy also resulted in the location of 33 QTLs responsible for differences in inflorescence architecture between foxtail and green millet (Doust et al. 2005) and 56 QTL loci contributing to differences in 15 morphological traits between closely related species of *Helianthus* (Avise 2004). Considering several taxa studied to date by QTL mapping, the composite number of genetic changes between even closely related species must normally be large. An important task will be to conduct similar kinds of QTL mapping experiments on closely related taxa, especially if their taxonomic status is controversial. A QTL-mapping approach is a method that could be used to identify chromosome regions responsible for taxonomic characters differentiating *L. multiflorum* and *L. perenne*. Even though several genetic maps of ryegrass have been reported, none included “speciation QTLs”. First, the genetic maps have been developed primarily for breeding purposes and therefore, nearly all of them have been published for the most important member of the genus, *L. perenne* (Bert et al. 1999; Armstead et al. 2002; Jones et al. 2002a; Yamada et al. 2004). Second, the majority of these maps have been based on the P150/112 reference mapping population consisted of about 150 individuals and derived by crossing a highly heterozygous *L. perenne* parent of complex descent, as a pollinator, with a doubled haploid

L. perenne female parent. This population, although useful for marker assisted selection, is not representative for the species. To increase the knowledge about genome structure and the evolutionary relationships between ryegrasses, the maps developed from different ryegrass populations, also different species should be established.

The wide spectrum of molecular methods available offers an exciting perspective to insight into practically each genome and its history. In many situations, they tell exactly the same story as morphological features. But in the others they disclose unusual relationships hidden up to the “molecular era”. Molecular methods are most useful when they address controversial areas such as within the genus *Lolium*. With species phylogeny properly sorted out via molecular methods, the origins of different *Lolium* phenotypes may become far more apparent. This is to say that molecular phylogenies may provide a road map of *Lolium* diversity.

2. GOALS

The aim of this research was to review the evolution within the genus *Lolium* using the broad spectrum of biometrical methods and high throughput molecular technologies. For the first time, the population genetic and phylogenetic methodologies were combined with genetic mapping in order to elucidate the relationships between two botanical species *L. multiflorum* and *L. perenne* and determine whether they can be regarded as distinct biological species. Besides commonly known technologies, own marker systems were offered. Additionally the other members of "Core Pooids" i.e., Poeae, Aveneae and Triticeae were compared with seven *Lolium* species in order to estimate the level of similarity between *Lolium* and the other representatives of grasses. To reach the overall goal the following objectives were set up:

2.1. To quantify the level of differentiation between two botanical taxa, L. *multiflorum* and *L. perenne*

Morphological variation in cultivars and ecotypes and influence of breeding activities on species differentiation were estimated using multivariate methods. A wide variety of molecular markers was used in order to estimate genetic variation and genetic similarity of both species. Moreover, the attempts were undertaken to clarify the early stages of evolution of *L. multiflorum* and *L. perenne* and their postglacial history based on transposons and organelle DNA. The origin of seedling root fluorescence in the genus *Lolium* was clarified.

2.2. To analsze the level of reproductive boundaries between *L. multiflorum* and *L. perenne* on the basis of genetic maps

To study a reproductive barrier formation and possibility of "sudden speciation" the level of marker distortions was compared in intra- and interspecific crosses. The genomic location of distorted regions was assessed with help of a genetic map constructed on the basis of F_2 population derived from *L. multiflorum* and *L. perenne*. A QTL mapping strategy was employed to identify cryptic variation at multiple genes responsible for taxonomic characters and to examine whether the variation at quantitative loci contributes to evolution of *L. multiflorum* and *L. perenne*.

2.3. To analyse evolutionary relationships within the genus *Lolium*

A battery of molecular methods was used to gain knowledge about evolutionary history of the genus *Lolium*. The data based on both nuclear and cytoplasmic data sets were compared and the consensus tree was drawn. Insertional polymorphism resulted from movements of different transposons was used to clarify the more ancient as well as the recent history of the genus. The division of the genus into two groups of autogamous and allogamous species was discussed in a view of own data and where necessary the revision of taxonomic status was proposed.

2.4. To analyse the relationships between the genus *Lolium* and the other representatives of the subfamily Pooideae

A large set of molecular data was used to address questions about genetic relatedness between the genus *Lolium* and the other members of Poeae, Avenae and Triticeae. The consistency of data from different nuclear sequences was checked. The possible role of introgression was discussed. The genetic map of *Lolium* was compared with the published maps of “Core Pooids”.

2.5. To estimate the efficiency of different marker systems for evolutionary studies

The efficiency, including costs, of several widely used marker systems such as RAPD, ISJ, AFLP as well as transposons and unique sequences based on nuclear and cytoplasmic DNA were compared. Moreover, the own marker system based on bacterial sequences was proposed for taxonomic and evolutionary studies in plants.

3. MORPHOLOGICAL VARIATION OF *L. MULTIFLORUM* AND *L. PERENNE*

3.1. INTRODUCTION

Separation of *L. multiflorum* and *L. perenne* is based on a number of morphological characters comprising vegetative, inflorescence and seed characters. Although morphological analyses are quite numerous, they concentrate either on classification of all species within the genus *Lolium* and the production of taxonomic keys or identification of cultivars within a species. A phenetic analysis has confirmed that individual characters do not separate clearly *L. multiflorum* and *L. perenne* so that a number of features have to be used together (Bennett 1997). Analysed characters are also not mutually exclusive for each species because their ranges overlap (Loos 1993a). As a result the position of *L. multiflorum* and *L. perenne* on dendrograms depends on characters applied and both species either can form a single cluster (Loos 1993a; Bennett et al. 2000) or belong to different clusters (Bennett 1997). The restraint of these data is also that a lot of them were obtained from herbarium specimens collected as wild ecotypes. This type of data usually informs only about total morphological variation with no idea which part of this variation is determined genetically. The majority of taxonomic characters are quantitative for which the genotype x environment interaction is observed. They can be related to the genotype only if species are compared in a field trial. Information on within species variation is only known for cultivars and breeding lines of perennial ryegrass (Fernando et al. 1997; Gilliland et al. 2000) and westerwold ryegrass landraces (Oliveira et al. 1997). To date however, there is a shortage of reliable field data regarding the morphological differentiation of *L. multiflorum* and *L. perenne*. Although some authors compared the morphology of both species in field experiments (Loos 1993a; Bennett et al. 2000) these studies used either a limited number of accessions or some characters were not included in the analysis. The usefulness of this information is also limited by one replication experiments and the different time of analysis applied for each species. Morphological divergence of closely related species can only be measured in detailed studies, such as this, where representative numbers of accessions including cultivars and ecotypes are analysed in replicated field trials. The aim of the present research was therefore, to evaluate morphological variation of *L. multiflorum* and *L. perenne* on the basis of replicated field experiment. The range of multivariate analyses was applied for clustering and measuring genetic distances between both species. Furthermore both species are subjected to intensive breeding activities aimed at high forage quality in the case of Italian ryegrass and high turf quality in the case of perennial ryegrass. These different goals can produce artificial differences not existing in nature. Thus, another goal was to compare cultivars and ecotypes to estimate the influence of human activity on species differentiation.

3.2. MATERIAL AND METHODS

In total 11 *L. multiflorum* (8 cultivars and 3 ecotypes) and 12 *L. perenne* (8 cultivars and 4 ecotypes) populations were used in morphological analyses. The origin of all populations and seed sources are listed in Annex 13.1. All experiments were conducted from 2002 to 2003. The overall experimental design is presented in Figure 13.1.1 (Annex 13.1). The fluorescence test was used to confirm the species identity (Annex 13.2). Seedling roots of *L. multiflorum* fluoresce when placed under UV light, and those of *L. perenne* do not fluoresce. From 20 to 30 genotypes were analysed per a population.

3.2.1. Analysed characters and sampling methods

The characters were selected on the basis of Terrell (1968), Loos (1993a) and Bennett (1997), plus following own observations. Where possible each plant was scored for 17 morphological characters. Seedling characters (leaf and root length) were scored together with seedling root fluorescence determination i.e., on the 14th day after germination. Adult vegetative characters were scored during two crops i.e., in July and September 2002 and then averaged. For each plant, three average-size basal leaves were removed from a plant and protected with a paper envelope. The leaf length and width were measured next day and the data were averaged for each plant. Flowering characters were studied during the second crop in July. Whenever it was possible the analysis was done both in 2002 and 2003 and then averaged. The plant height was determined before cut. The three well developed spikes together with flag leaves were collected from each plant, placed in a paper bag and used for spike, spikelet and floret character measurements. The areas of vegetative and flag leaves were counted by multiplication of the leaf length by leaf width. The green and dry weight were measured twice i.e., in July and September. Recovery after winter was analysed as a number of plants survived for which at least 50% of a tuft started to grow and developed into mature plants.

3.2.2. Statistical methods

Data were analysed using STATISTICA 7.1 software. Range of variation was plotted on the basis of maximum and minimum values and standard deviation. Analysis of variance was used to check for population, species, form (cultivar or ecotype) and interaction species x form effects. The LSD test (Least Significant Difference) was used to examine differences between all possible pairs of means (multiple comparisons). Each population was further characterized by main effects i.e., deviation of its mean from the total mean per a species. The F statistics was used for estimation of the significance of main effects.

The analysis of discriminant value was employed to estimate the participation of each character in total variation of analysed populations. The discriminant value of each character was estimated by partial lambda Wilks statistics. It has range from 0 to 1 and the lower lambda Wilks statistics the higher discriminant value of a character. Thus, the value 1.00 means that a character has no discriminant value. The discriminant analysis was made separately

for species, forms (ecotypes, cultivars) and populations. A number of multivariate analyses were used to classify and group all the populations, and as each method is designed to sort the data on different bias, they were used comparatively to verify the relationships found. The observations per plant were used for canonical variate analysis (CVA) to estimate the similarity between populations on the basis of all characters together. Species, forms and populations were used as grouping variables. The final squared Mahalanobis distance (D^2) was estimated between *L. multiflorum* and *L. perenne* as well as between all the populations and used to express the similarity. The canonical variate coefficients were used to cluster the populations. The Euclidean distance and Ward's method were applied for clustering. The transformed data were also used for calculation of input distance matrix for multidimensional scaling.

The characters were standardized across populations, and population averages were calculated for principal component analysis (PCA). The original characters were transformed into a set of new, uncorrelated variables arranged in order of decreasing importance. Principal component and factor analysis was used to identify patterns in data. PCA was performed on correlation matrix to reduce all the data to a common scale. The number of factors was extracted on the basis of eigenvalues using the Kaiser criterion. The highest principal components were used to draw scatterplots of all populations.

3.3. RESULTS

3.3.1. Discriminant values of analysed characters

There was a wide range of variation across *L. multiflorum* and *L. perenne* for all analysed characters. Examination of the highest and lowest values revealed that only the 1^{sr} year ear emergence separated clearly both species (Figure 3.1R). Most characters frequently used in taxonomy i.e., basal and flag leaf length, width and area, culm height at ear emergence, spikelet length and floret number did not differentiate Italian and perennial ryegrass because their variation ranges overlapped (Figure 3.1C-E; H; J; L; M-O). Similarly, the overlapping variation was observed in seedling length, green and dry weight (Figure 3.1A; F-G). The hiatus between both species was hardly observed even in recovery after winter (Figure 3.1P) that is traditionally regarded as the most discriminant character. However, the populations of *L. multiflorum* with as much as 80% of surviving plants in the 2nd year of cultivation were observed as well as the populations of *L. perenne* with only 30% of recovered plants were found.

The difficulties in separation of ryegrasses on the basis of studied characters were also confirmed by their discriminant values (Table 3.1-3.2). The 1st year ear emergence was the only character that separated either *L. multiflorum* from *L. perenne* or cultivars from ecotypes as estimated by low partial lambda Wilks statistics (0.369 and 0.379, respectively). In the case of species, from remaining 4 characters included in the 1st discriminant variable, only dry weight had certain discriminant value (lambda Wilks statistics equal 0.788). The lambda

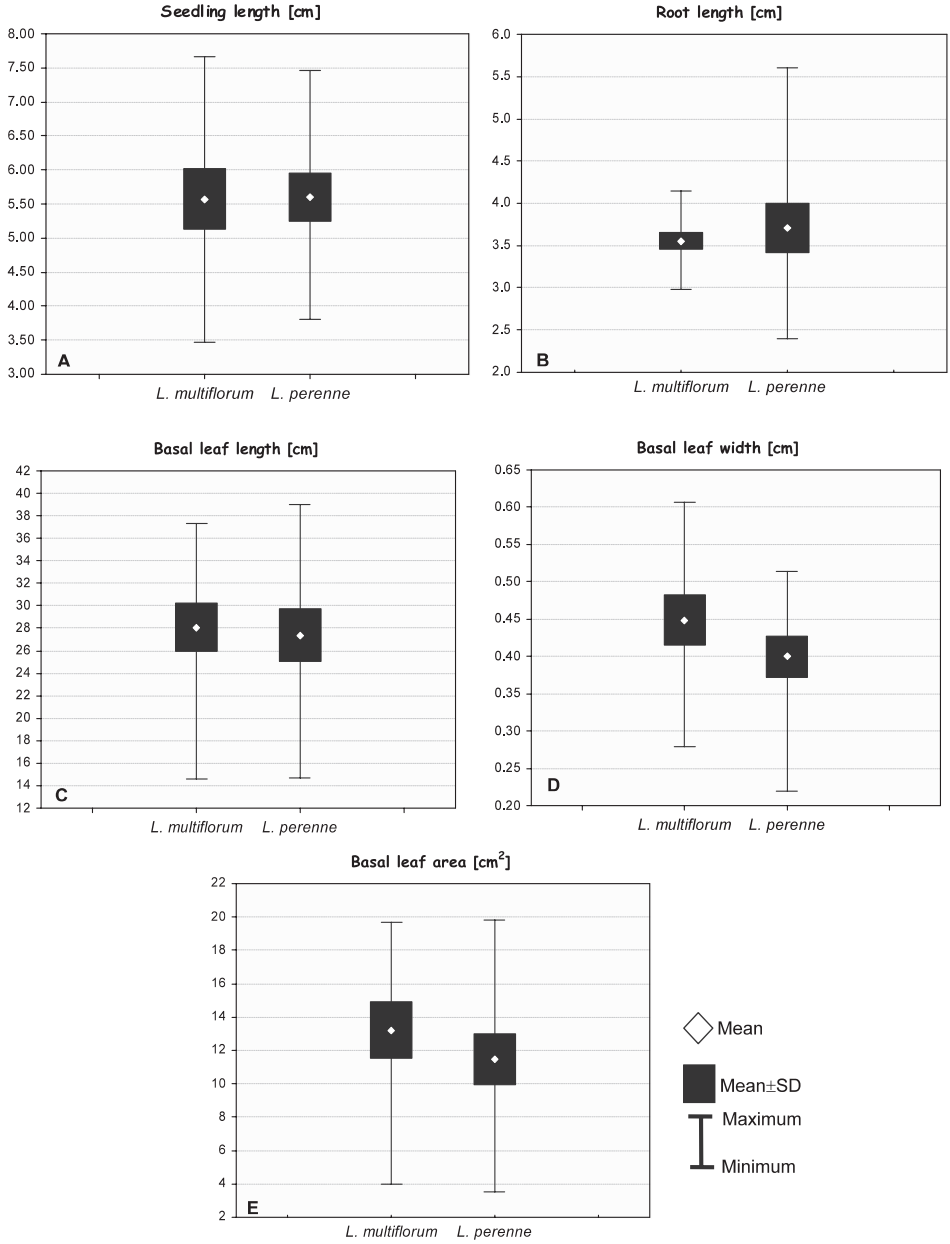


Figure 3.1. Range of variation of morphological characters in *L. multiflorum* and *L. perenne*

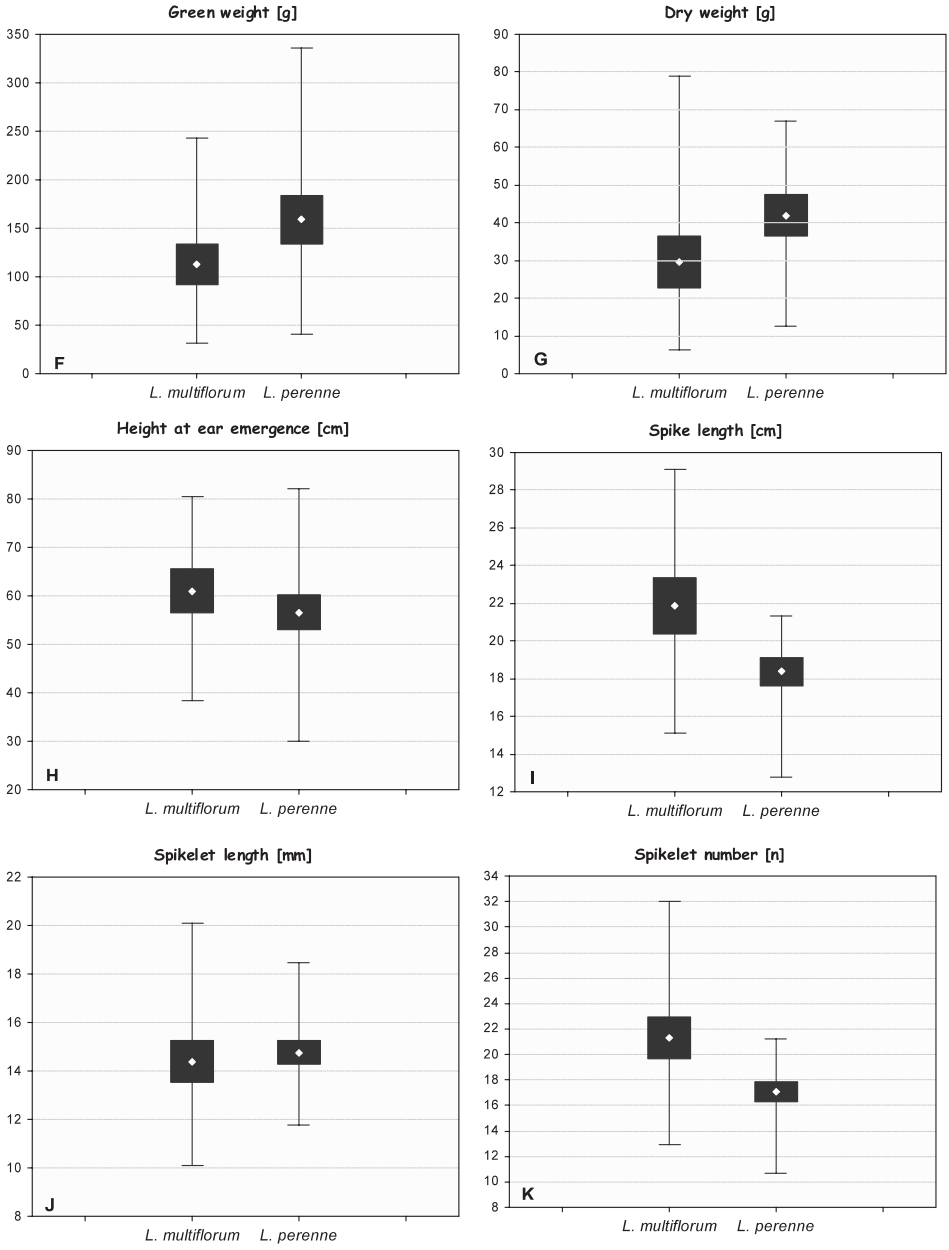


Figure 3.1. Range of variation of morphological characters in *L. multiflorum* and *L. perenne*, continued

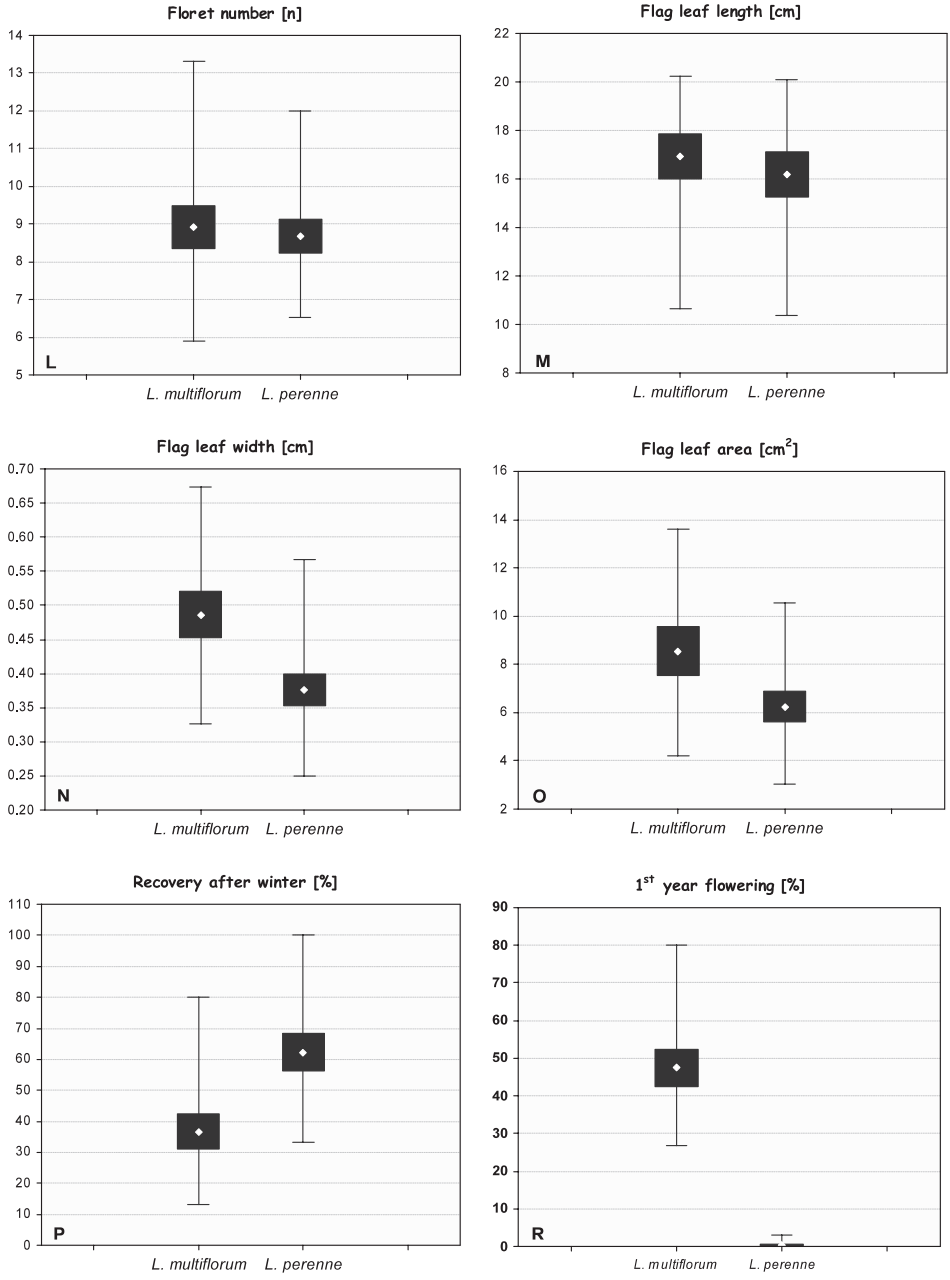


Figure 3.1. Range of variation of morphological characters in *L. multiflorum* and *L. perenne*, continued

Wilks statistics close to 1.00 suggested that species separation was rather impossible on the basis of flag leaf width, spike length and basal leaf area (Table 3.1). The distinction of *L. multiflorum* and *L. perenne* based on cultivars and ecotypes was much easier and could be done with help of 2 discriminant variables (variable 1 and 2) that explained 96% of observed variation. The most important characters, besides the 1st ear emergence included seedling leaf and recovery after winter (Table 3.2). Moderate discriminant values were also revealed by flag leaf width, basal leaf area and height at ear emergence. Dry, green weight and spike length appeared to be fairly minor characters (lambda Wilks close to 1.00). By contrary, all 23 populations were found to differ from each other in nine discriminant variables that explained 95% of observed variation. Most of analysed characters showed high discriminant value with lambda Wilks statistics of 0.19 to 0.36 (Table 3.3). The highest discriminant values were shown by seedling leaf and height at ear emergence while the lowest significant value (0.411) was observed for flag leaf length. But still it was almost twice as high as for species. Interestingly, there was not possible to distinguish all analysed populations on the basis of plant weight (green and dry).

Table 3.1. Discriminant value of morphological characters in *L. multiflorum* and *L. perenne*

Character	Lambda Wilks (Partial)	F statistics	χ^2 for 1st discriminant variable
I st year ear emergence	0.369	107.8	χ^2=91.63
Dry weight	0.788	16.9	
Flag leaf width	0.945	3.7	
Spike length	0.945	3.7	
Basal leaf area	0.958	2.8	
Critical value, P=0.05		$F_{0.05}$=4.00	χ^2=11.07

3.3.2. Comparison of *L. multiflorum* and *L. perenne*

Analysis of variance indicated that species effect was not significant at P=0.05 for about 60% of studied characters (Table 3.4). However, the UPOV (1991) recommends standards for "Distinctiveness" in testing of ryegrasses at P level equivalent to 0.01. Using the P=0.01 standard, *L. multiflorum* and *L. perenne* were not distinct in 76% of characters. Examination of mean values for each species showed that they were very alike for about 35% characters including these used in taxonomic classification such as basal and flag leaf length, spikelet length and floret number (Table 3.5).

The significant differences between species means were found for eight (40%) studied characters at P=0.05. When P=0.01 was taken, the differences were found for four characters (24%) including flag leaf width and area, recovery after winter and the 1st year ear emergence (Table 3.5). As expected, the greatest separation between *L. multiflorum* and *L. perenne* was observed in the 1st year ear emergence and recovery after winter. In the first year of cultivation ear emergence was observed in 42% of Italian ryegrass plants. On opposite, *L. perenne* did not flower during the sowing year with exception of several plants from New *Zea*land ecotype (Table 3.5). In the second year of cultivation surprisingly high number of *L. multiflorum* plants survived and reached full maturity (36%), however almost double the

Table 3.2. Discriminant value of morphological characters in cultivars and ecotypes of *L. multiflorum* and *L. perenne*

Character	Lambda Wilks (Partial)	F statistics	χ^2 for discriminant variables		
			1st	2nd	3rd
I st year ear emergence	0.379	31.01			
Seedling leaf length	0.537	16.38			
Recovery after winter	0.685	8.73			
Flag leaf width	0.825	4.02			
Basal leaf area	0.839	3.66	χ^2=186.53	χ^2=81.11	χ^2=16.18
Height at ear emergence	0.852	3.29			
Dry weight	0.903	2.05			
Spike length	0.915	1.77			
Green weight	0.942	1.18			
Critical value, P=0.05		$F_{0.05}$=2.79	χ^2=40.11	χ^2=26.29	χ^2=14.07

Table 3.3. Discriminant value of morphological characters in 23 populations of *L. multiflorum* and *L. perenne*

Character	Lambda Wilks (Partial)	F statistics	χ^2 for discriminant variables		
Seedling leaf length	0.145	8.01	1st	2nd	3rd
Height at ear emergence	0.153	7.58			
Basal leaf length	0.188	5.91			
Spikelet length	0.191	5.77	1 144.88	968.77	814.83
Spikelet number	0.198	5.54			
Basal leaf width	0.240	4.31			
Recovery after winter	0.264	3.80	4rd	5th	6th
I st year ear emergence	0.285	3.42			
Basal leaf area	0.292	3.31			
Root length	0.318	2.92	684.77	562.76	466.09
Flag leaf length	0.344	2.59			
Floret number	0.362	2.40			
Flag leaf width	0.411	1.96	7th	8th	9th
Spike length	0.446	1.69			
Green weight	0.449	1.68	385.44	308.33	240.88
Flag leaf area	0.457	1.62			
Dry weight	0.494	1.39			
		$F_{0.05}$=1.93	χ^2=43.77		

number of such plants was observed in *L. perenne*. There was much less variation between both species in the remaining 6 characters. Generally *L. multiflorum* expressed higher mean values than *L. perenne* with the only exception of green and dry weight.

To help interpret the nature of these differences between *L. multiflorum* and *L. perenne*, cultivars and ecotypes of both species were compared. Analysis of variance revealed significant interaction species x form for two characters that suggested different behavior of culti-

Table 3.4. Statistics F for main effects and interaction for analysed characters in *L. multiflorum* and *L. perenne*

Character	Source of variation			
	Population	Species	Form (cultivar/ecotype)	Species x Form
Seedling leaf length	**13.09**	0.44	**40.55**	0.25
Seedling root length	5.54	0.41	1.97	0.04
Basal leaf length	**30.69**	0.37	**6.73**	1.09
Basal leaf width	**8.8**	**4.90**	3.50	2.70
Basal leaf area	**18.00**	3.00	**6.08**	2.55
Green weight	**9.59**	3.22	2.60	0.93
Dry weight	**4.98**	3.17	3.78	0.58
Height at ear emergence	**21.54**	0.04	**5.01**	**9.77**
Spike length	**8.26**	**6.81**	2.67	0.88
Spikelet length	**15.47**	1.23	1.57	1.34
Spikelet number	**13.58**	**8.75**	**6.11**	2.12
Floret number	2.43	0.05	1.08	1.14
Flag leaf length	**4.98**	0.06	2.86	1.19
Flag leaf width	**8.60**	**10.32**	**16.18**	**4.85**
Flag leaf area	**6.55**	**4.09**	**11.06**	3.93
Recovery after winter	**9.62**	**23.54**	**22.75**	0.07
Ist year ear emergence	**8.08**	**94.99**	1.72	2.19
F critical value for P=0.05 P=0.01	**1.74 2.18**		**3.98 7.01**	

vars and ecotypes (Table 3.4). For example the cultivars of *L. multiflorum* had much wider flag leaves than ecotypes. But in *L. perenne*, no differences between cultivars and ecotypes were found in the flag leaf width. Examination of mean values for cultivars and ecotypes showed clearly that similar relationship was observed for other characters differed between both species (Table 3.5). The most frequently higher values appeared to be present in Italian ryegrass cultivars. They were higher, had longer spikes, more spikelets, wider flag leaves with bigger area in comparison with ecotypes of this species. This is likely to be because of their very intensive selection during breeding programmes directed to production of forms for hay and silage. Interestingly, no differences were observed for these characters between ecotypes of *L. multiflorum* and *L. perenne*. Opposite relationships were observed for green and dry weight. They had the greatest values in *L. perenne* cultivars while ecotypes of both species did not differ significantly.

As confirmation of cultivar separation Mahalanobis distances were computed for all groups. Ecotypes of *L. multiflorum* and *L. perenne* appeared the most similar as shown by the lowest distance between them (Table 3.6). Morphology of *L. perenne* cultivars was not changed very much in comparison with ecotypes because distance between them was only a slightly higher. However, the high distance between *L. multiflorum* cultivars and the remaining groups appeared to confirm the separation of the former.

Table 3.5. Mean values of 17 morphological characters in *L. multiflorum* and *L. perenne*

Character	*L. multiflorum*		*L. perenne*	
	Cultivars	Ecotypes	Cultivars	Ecotypes
Seedling leaf length [cm]±SD	6.13±1.41[a]	4.08±0.51[b]	6.18±1.14[a]	4.43±0.72[b]
	5.57±1.53		5.60±1.31	
Seedling root length [cm]±SD	3.63±0.70	3.36±0.54	3.83±1.10	3.45±0.83
	3.56±0.66		3.70±1.02	
Basal leaf length [cm]±SD	28.88±6.77[a]	25.89±8.72[ab]	29.73±7.32[a]	22.71±7.35[b]
	28.10±7.32		27.40±7.97	
Basal leaf width [cm]±SD	0.45±0.11[a]	0.44±0.15[a]	0.43±0.11[a]	0.33±0.05[b]
	0.45±0.12		0.40±0.11	
Basal leaf area [cm^2]±SD	13.54±5.61[a]	12.34±6.53[ab]	13.35±5.21[a]	7.72±3.19[b]
	13.21±5.79		11.48±5.32	
Green weight [g]±SD	116.69±71.18[b]	100.27±100.25[b]	178.07±97.50[a]	120.71±65.67[ab]
	112.75±78.72[b]		**158.95±91.40[a]**	
Dry weight [g]±SD	31.55±24.70[b]	23.99±26.45[b]	47.77±25.67[a]	30.51±17.57[b]
	29.49±25.00[b]		**42.01±24.45[a]**	
Height at ear emergence [cm]±SD	65.98±13.57[a]	47.76±11.67[b]	55.57±13.97[b]	58.59±9.55[ab]
	61.01±15.30		58.58±12.61	
Spike length [cm]±SD	22.66±5.44[a]	19.76±5.54[ab]	18.65±2.89[b]	17.87±2.63[b]
	21.87±5.54[a]		**18.39±2.79[b]**	
Spikelet length [mm]±SD	14.81±3.02	13.21±2.68	14.78±2.31	14.72±1.52
	14.38±2.98		14.75±2.06	
Spikelet number [n]±SD	22.54±6.33[a]	18.02±2.02[b]	17.45±3.34[b]	16.29±1.57[b]
	21.30±5.83[a]		**17.07±2.91[b]**	
Floret number [n]±SD	9.28±1.98	8.00±1.85	8.67±3.01	8.69±1.20
	8.93±2.00		8.68±2.53	
Flag leaf length [cm]±SD	17.66±3.99	15.03±2.39	16.39±3.23	15.83±4.11
	16.94±3.78		16.20±3.49	
Flag leaf width [cm]±SD	0.53±0.12[a]	0.37±0.04[b]	0.39±0.10[b]	0.35±0.05[b]
	0.49±0.12[a]		**0.38±0.09[b]**	
Flag leaf area [cm^2]±SD	9.66±3.83[a]	5.59±1.17[b]	6.59±2.66[b]	5.56±1.86[b]
	8.55±3.78[a]		**6.25±2.45[b]**	
Recovery after winter [%]	30.50[c]	53.53[b]	53.75[b]	79.30[a]
	36.73[b]		**62.27[a]**	
Ist ear emergence [%]	58.87[a]	38.21[a]	0.00[b]	0.77[b]
	42.42[a]		**0.26[b]**	

[abc]different letters mean significant differences at P=0.05 for LSD test

Table 3.6. The squared Mahalanobis distances between cultivars and ecotypes of *L. multiflorum* and *L. perenne*

Form	*L. multiflorum* cultivars	*L. multiflorum* ecotypes	*L. perenne* cultivars	*L. perenne* ecotypes
L. multiflorum cultivars	0.00	13.06	21.32	26.05
L. multiflorum ecotypes		0.00	16.48	8.29
L. perenne cultivars			0.00	10.80
L. perenne ecotypes				0.00

$F_{0,05}$= 2.07

3.3.3. Comparison of *L. multiflorum* and *L. perenne* populations

For the majority of characters there was significant variation between populations. Among analysed characters, basal leaf length and area differed from the species mean in the majority of populations (Table 3.7-3.8). Almost no deviation from a mean was observed in the 1st year ear emergence. Examination of the highest and lowest values for each character revealed that some cultivars were frequently expressing values at the outer limits of the character ranges (Table 3.7). In *L. multiflorum*, Bartissimo was at the upper extremities of the range in seven characters, following by Nagamamikari that was the greatest for four characters. Solen was the most extreme *L. perenne* cultivar as confirmed by the highest values in ten characters. The lowest extremities were occupied by Asso and Italian ecotype (*L. multiflorum*) and Kyosato (*L. perenne*). The estimation of main effects confirmed that cultivars generally expressed higher values than the mean for a given species while lower values were observed in ecotypes of both species (Table 3.8). The only exception was the recovery after winter for which the highest values were observed in *L. perenne* ecotypes while cultivars of this species were less winter hardy. The fewest plants surviving the winter were found in Japan cultivar Kyosato (33%) and surprisingly, Polish line Ba012 (38%). Italian ecotype of *L. multiflorum* and New *Zea*land ecotype of *L. perenne* displayed significantly lower values than a species mean for the majority of studied characters. Interestingly, both ecotypes expressed some unusual deviations from the mean, as compared with the species. Italian ecotype of *L. multiflorum* was characterized by relatively high recovery after winter; about 80% plants survived and reached full maturity. New *Zea*land ecotype was the only population of *L. perenne* with some plants (3%) flowered during the 1st year of cultivation.

No clear separation of *L. multiflorum* and *L. perenne* populations was obtained when clustering included all studied characters (Figure 3.2). The phenogram based on squared Mahalanobis distances consisted from two major clusters containing populations from both species. The first cluster (cluster 1) consisted of *L. multiflorum* and *L. perenne* cultivars expressing lower green and dry weight in comparison with species means. The second cluster (cluster 2) consisted of *L. multiflorum* ecotypes (German, Spanish) and *L. perenne* populations (cultivars and ecotypes) with average values for the majority of characters. These two clusters were grouped together and then they joined with a cluster of three, high yielding *L. multiflorum* cultivars (Bartissimo, Crema, Nagamamikari) and Solen, *L. perenne* cultivar of the highest plant weight (cluster 3). The most outlying group was formed from populations characterized by the lowest values of most studied characters (*L. multiflorum* - Italian ecotype; *L. perenne* - Kyosato, NGB5036, New *Zea*land ecotype).

Table 3.7. Mean values for 17 morphological characters in cultivars and ecotypes of *L. multiflorum* and *L. perenne*

Species	Population	Seedling leaf length [cm]	Seedling root length [cm]	Basal leaf length [cm]	Basal leaf width [cm]	Basal leaf area [cm^2]	Green weight [g]	Dry weight [g]	Height at ear emergence [cm]	Spike length [cm]	Spikelet length [mm]	Spikelet number [n]	Floret number [n]	Flag leaf length [cm]	Flag leaf width [cm]	Flag leaf area [cm^2]	Recovery after winter [%]	1st year ear emergence [%]
L. multiflorum	**Asso**	6.62	3.75	24.87	0.47	11.84	115.38	40.40	57.36	**15.13**	11.59	**12.97**	8.61	15.93	0.45	7.10	**13.33**	74.50
	Atalja	5.27	3.67	25.03	0.47	11.63	56.84	9.96	41.57	16.39	13.65	17.31	8.98	**10.66**	0.40	**4.42**	30.00	50.32
	Bartissimo	7.27	4.14	34.72	0.51	17.80	243.18	78.86	80.28	23.30	13.43	28.14	9.11	20.22	0.67	13.61	17.67	80.00
	Bartolini	7.67	3.43	19.88	**0.28**	5.70	87.86	19.44	69.17	22.73	18.28	21.33	8.70	17.33	0.46	8.10	56.33	41.00
	Crema	7.54	3.43	32.56	0.48	15.86	188.24	52.48	80.53	23.45	12.19	21.25	6.54	19.90	0.60	12.02	36.67	49.43
	Limulta	5.81	3.27	33.92	0.58	19.68	**31.40**	**6.40**	58.63	28.08	14.67	31.99	8.89	17.33	0.47	8.48	20.00	48.41
	Nagamamikari	4.70	3.72	37.30	0.50	18.63	132.60	25.00	75.20	29.10	20.10	21.40	13.30	20.10	0.62	12.44	40.00	36.67
	Waseaoba	4.17	3.60	22.80	0.31	7.13	78.00	19.90	65.10	23.10	14.60	25.90	10.10	19.80	0.57	11.29	**30.00**	**26.67**
	German (E)	**3.48**	**2.98**	33.30	0.45	14.88	58.43	10.23	**38.30**	16.27	14.22	16.22	9.11	14.01	0.38	5.28	40.00	30.90
	Italian (E)	4.20	3.30	**14.57**	**0.28**	**4.01**	192.17	48.57	43.17	17.08	**10.12**	18.27	**5.90**	14.62	**0.33**	4.80	80.00	40.67
	Spanish (E)	4.57	3.81	29.81	0.61	18.12	56.20	13.17	61.80	25.91	15.28	19.56	9.00	16.46	0.41	6.69	40.00	43.07
L. perenne	**Argona**	6.61	3.20	29.44	0.34	10.11	231.51	66.69	55.42	21.24	18.46	15.98	10.04	11.60	0.35	4.01	65.00	0.00
	Ba012	5.81	3.62	33.60	0.50	16.70	97.44	26.32	**30.00**	17.90	**12.73**	21.22	**6.53**	17.56	0.42	7.32	38.33	0.00
	Kyosato	5.88	3.50	15.97	**0.22**	**3.52**	**40.40**	25.77	56.00	**12.77**	15.33	**10.67**	8.00	12.10	**0.25**	**3.04**	**33.33**	0.00
	Lisuna	7.37	5.44	32.00	0.48	15.49	97.03	24.90	55.33	18.02	11.75	19.72	7.00	17.39	0.43	7.49	60.00	0.00
	Magella	5.59	3.63	21.47	0.48	10.29	192.86	52.99	54.17	18.01	16.22	17.30	9.33	15.78	0.40	6.37	48.33	0.00
	Merganda	4.43	2.44	32.30	0.45	14.50	197.72	59.86	54.17	20.36	14.97	16.93	6.81	20.08	0.42	8.42	55.00	0.00
	Rela	7.47	5.61	34.00	0.48	16.35	231.57	58.66	57.50	19.59	14.78	19.16	9.67	18.30	0.30	5.49	75.00	0.00
	Solen	6.28	3.20	39.04	0.51	19.84	336.02	66.95	82.00	21.33	14.00	18.67	12.00	18.33	0.57	10.57	55.00	0.00
	NGB5030 (E)	4.81	3.53	29.60	0.34	10.07	106.30	23.50	68.33	18.37	15.33	17.67	8.90	19.30	0.37	7.10	100.00	0.00
	NGB5036 (E)	3.91	**2.40**	**14.67**	0.34	5.01	127.75	33.55	51.17	20.27	15.33	15.43	9.43	15.40	0.36	5.57	88.33	0.00
	Hungarian (E)	5.20	4.48	29.77	0.38	11.31	203.83	52.30	66.07	18.68	14.15	16.98	8.83	18.25	0.34	6.20	83.45	0.00
	New Zealand (E)	**3.80**	3.40	16.80	0.27	4.48	44.96	**12.68**	48.78	14.15	14.05	15.07	7.60	**10.36**	0.32	3.36	45.43	3.07

(E) – ecotypes; **shadow boxes –** maximum values for each species, **bold –** minimum values for each species

Table 3.8. Estimation of main effects for morphological characters in *L. multiflorum* and *L. perenne*

Species	Population	Seedling leaf length	Seedling root length	Basal leaf length	Basal leaf width	Basal leaf area	Green weight	Dry weight	Height at ear emergence	Spike length	Spikelet length	Spikelet number	Floret number	Flag leaf length	Flag leaf width	Flag leaf area	Recovery after winter	1st year ear emergence
L. multiflorum	**Asso**	0.68	0.29	-0.44	0.15	-0.24	0.03	0.44	-0.24	**-1.22**	**-0.94**	**-1.43**	-0.16	-0.27	-0.32	-0.38	**-1.15**	1.08
	Atalja	-0.19	0.17	-0.41	0.18	-0.27	-0.71	-0.78	**-1.27**	**-0.99**	-0.24	-0.68	0.02	**-1.66**	-0.72	**-1.14**	-0.33	0.11
	Bartissimo	1.11	0.89	0.90	0.53	0.79	1.66	1.97	1.26	0.26	-0.32	1.17	0.09	0.87	1.50	1.34	**-0.93**	1.29
	Bartolini	1.36	-0.19	**-1.12**	**-1.35**	**-1.29**	-0.32	-0.40	0.53	0.16	1.31	0.01	-0.11	0.10	-0.18	-0.12	0.96	-0.26
	Crema	1.28	-0.19	0.61	0.26	0.46	0.96	0.91	1.27	0.29	-0.73	-0.01	**-1.19**	0.78	0.88	0.92	-0.00	0.08
	Limulta	0.16	-0.43	0.79	1.08	1.12	-1.03	**-0.92**	-0.16	1.12	0.09	1.83	-0.02	0.10	-0.15	-0.02	-0.82	0.04
	Nagamamikari	-0.56	0.25	1.26	0.42	0.94	0.25	-0.18	0.92	1.31	1.92	0.02	2.18	0.84	1.07	1.03	0.16	-0.43
	Waseaoba	**0.91**	0.07	**-0.72**	**-1.13**	**-1.05**	-0.44	-0.38	0.27	0.22	0.08	0.79	0.58	0.76	0.67	0.73	-0.33	-0.83
	German (E)	**-1.37**	-0.87	071	-0.01	0.29	-0.69	-0.77	**-1.48**	**-1.01**	-0.06	**-0.87**	0.09	-0.78	-0.85	-0.87	0.16	-0.66
	Italian (E)	**-0.89**	-0.39	**-1.84**	**-1.41**	**-1.59**	1.01	0.76	**-1.17**	-0.86	**-1.43**	-0.52	**-1.51**	-0.62	**-1.28**	-0.99	2.12	-0.27
	Spanish (E)	**-0.65**	0.38	0.24	1.29	0.85	-0.72	-0.65	0.05	0.73	0.30	-0.29	0.03	-0.13	-0.64	-0.49	0.16	-0.17
L. perenne	**Argona**	0.77	-0.49	0.25	-0.53	-0.26	0.79	1.01	-0.09	1.02	1.79	-0.37	0.54	**-1.31**	-0.32	-0.91	0.12	-0.27
	Ba012	0.16	-0.08	0.77	0.94	0.98	-0.67	-0.64	**-2.11**	-0.18	-0.99	1.42	-0.85	0.39	0.44	0.44	**-1.02**	-0.27
	Kyosato	0.21	-0.20	**-1.43**	**-1.69**	**-1.49**	**-1.29**	-0.66	-0.05	**-2.01**	0.28	**-2.20**	-0.27	**-1.17**	**-1.39**	**-1.31**	**-1.23**	-0.27
	Lisuna	1.35	1.69	0.57	0.78	0.76	-0.68	-0.69	-0.09	-0.13	**-1.46**	0.91	-0.66	0.33	0.59	0.51	-0.09	-0.27
	Magella	-0.01	-0.08	**-0.74**	0.75	-0.22	0.37	0.45	-0.19	-0.14	0.71	0.08	0.26	-0.12	0.29	0.05	-0.59	-0.27
	Merganda	**-0.89**	**-1.23**	0.61	0.47	0.56	0.42	0.72	-0.19	0.70	0.10	-0.05	-0.74	1.11	0.48	0.89	-0.31	-0.27
	Rela	1.43	1.85	0.83	0.75	0.91	0.79	0.68	0.07	0.43	0.01	0.72	0.39	0.59	-0.84	-0.31	0.54	-0.27
	Solen	0.52	-0.49	1.46	1.07	1.57	1.93	1.02	2.02	1.05	-0.37	0.55	1.31	0.61	2.11	1.76	-0.31	-0.27
	NGB5030 (E)	-0.59	-0.17	0.28	-0.56	-0.26	-0.58	-0.76	0.93	-0.01	0.28	0.21	0.09	0.88	-0.11	0.35	1.60	-0.27
	NGB5036 (E)	**-1.29**	-1.27	**-1.59**	-0.56	**-1.22**	-0.34	-0.35	-0.43	0.67	0.28	-0.56	0.29	-0.23	-0.22	-0.27	1.11	-0.27
	Hungarian (E)	-0.30	0.76	0.29	-0.19	-0.03	0.49	0.42	0.75	0.10	-0.29	-0.03	0.06	0.59	-0.42	-0.02	0.90	-0.27
	New Zealand (E)	**-1.37**	-0.29	**-1.33**	**-1.25**	**-1.49**	**-1.25**	-1.19	**-0.62**	**-1.52**	-0.34	-0.69	-0.43	**-1.67**	-0.62	**-1.18**	-0.72	2.94

(E) – ecotypes; **shadow boxes –** significantly higher values than a species mean, **bold –** significantly lower values than a species mean at P=0.05

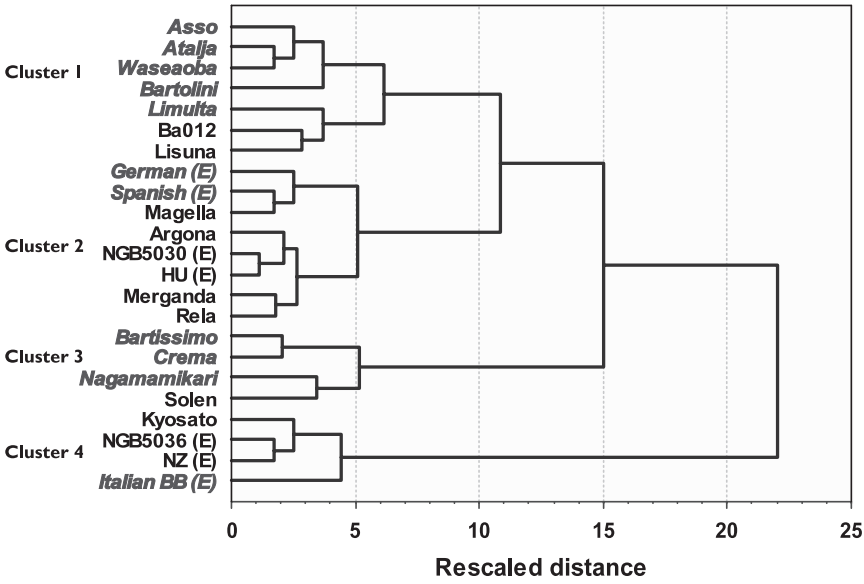


Figure 3.2. Phenogram based on the squared Mahalanobis distance between 23 populations, clustered usig Ward's method

Principal component analysis (PCA) was found to explain 87% of the variation between populations in the six components (Table 3.9). The first two principal components (PC1, PC2) explained 53% of the variation and they are concerned with generative characters (height at ear emergence, spike length, spikelet number and flag leaf) and plant weight (dry and green). This analysis showed that two characters commonly regarded as highly discriminant for *L. multiflorum* and *L. perenne* i.e., winterhardiness and the 1st year ear emergence explained only 6% of the variation between populations (PC6). Following PCA, as in clustering based on the Mahalanobis distance, the separation of *L. multiflorum* and *L. perenne* was not possible. Plots of the first two principal components separated well only six cultivars of *L. multiflorum* (Figure 3.3A) that had longer spike, greater number of spikelets and bigger flag leaf (longer, wider and bigger area). The ecotypes of *L. multiflorum* were placed among *L. perenne* populations. Furthermore, the overlapping was even higher when additional generative characters were added (spikelet length and floret number) as it was shown by the scatterplot based on PC1 and PC3. These two functions separated Crema, Bartissimo in addition to Nagamamikari that occupied different position probably because of higher florets number than other *L. multiflorum* populations (Figure 3.3B). A slightly better separation was received by PC2 and PC4 concerned with vegetative characters (Figure 3.3C). In this plot most *L. multiflorum* populations formed a fairly separate cluster and high yielding *L. perenne* (Rela, Solen, Argona) were grouped together. Moreover, low yielding *L. multiflorum* populations (Atalja, Limulta) were placed apart from *L. perenne* populations. However, these two variables were only responsible for 24% of variation and there was still some overlapping in the middle of the scatterplot. Because of difficulties in separation of *L. multiflorum* and *L. perenne*, a multidimensional scaling based on distance matrix was performed (Figure 3.4). When tree dimensions were defined the better degree of separation was obtained. Follow-

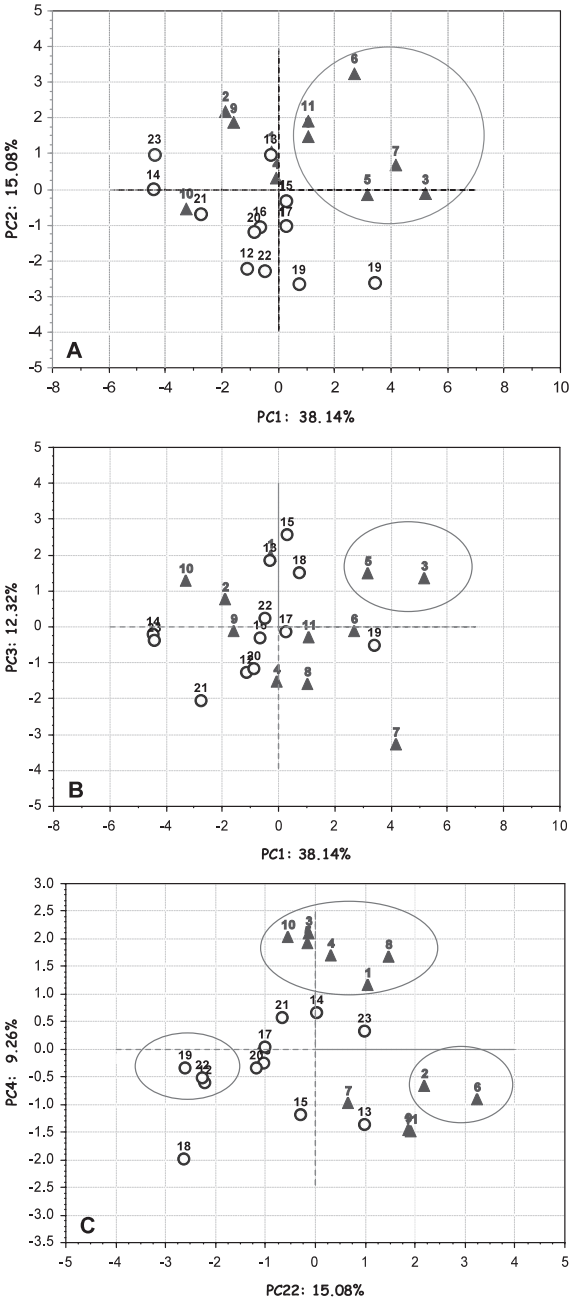
ing additional rotation the majority of *L. multiflorum* cultivars formed a fairly separate cluster which did not overlap. However, *L. multiflorum* ecotypes were still more closely associated with *L. perenne*. For example, ecotypes from Germany and Spain together with Japan cultivar Nagamamikari formed a group with *L. perenne* cultivars (Ba012, Magella, Merganda). Moreover, the Italian ecotype was still linked with *L. perenne* ecotypes.

Table 3.9. Principal component scores with corresponding variation

Variation	Principal component					
	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	6.48	2.56	2.09	1.57	1.14	1.05
Percentage variation [%]	38.14	15.08	12.32	9.26	6.71	6.19
Cumulative variation [%]	38.14	53.22	65.54	74.80	81.51	87.70
Original characters with the highest loadings	Height 0.65 Spike length 0.74 Spikelet number 0.76	Green weight 0.65	Spikelet length 0.91	Basal leaf length 0.85	Seedling leaf 0.78	Recovery after winter -0.87
	Flag leaf length 0.83 Flag leaf width 0.75	Dry weight 0.95	Floret number 0.87	Basal leaf width 0.90 Basal leaf area 0.93	Root length 0.88	1st year ear emergence 0.81
	Flag leaf area 0.86					

3.4. DISCUSSION

Good biological species are characterized by easily recognised morphological features. Even closely related species can be distinguished without doubts. The inbreeding ryegrasses are clearly separated from the cross-breeders by several characters. In general, the inbreeding populations are smaller, contain fewer florets, and the ears emerge earlier (Loos 1993a). Persian darnel, *L. persicum* can be distinguished by extremely long awns not present in any other *Lolium* species. Beside the fact that *L. remotum* reveals a wide variation that in many cases overlaps the ranges of the other species (Bennett 1997) it is recognized by small, barrel like seeds. On the other hand *L. temulentum* has quite large "cereal" like seeds. These seed characters are often used as determination characters for inbreeding species (Terrell 1968). In contrast, the three cross-breeding *Lolium* species are very similar and their populations often intergrade. It appears from various methods of multivariate analysis employed in this study that *L. multiflorum* and *L. perenne* resemble each other. Most morphological characters are very alike in both species and all but the 1st year ear emergence have a very low discriminant value. The strong influence of ear emergence as a criterion for differentiation has been previously reported by Bennett et al. (2000). Most *L. perenne* accessions never flower without low temperature exposure, and therefore, a late spring sowing is an efficient method of separating *L. multiflorum* from *L. perenne* populations. Although this character is useful for a breeding purpose, it is useless for classification of wild ecotypes. It is not possible to recognize whether a particular



L. multiflorum
1. Asso
2. Atalja
3. Bartolini
4. Bartissimo
5. Crema
6. Limulta
7. Nagamamikari
8. Waseaoba
9. German ecotype
10. Italian ecotype
11. Spanish ecotype

L. perenne
12. Argona
13. Ba012
14. Kyosato
15. Lisuna
16. Magella
17. Merganda
18. Rela
19. Solen
20. Ngb5030
21. NGB5036
22. Hungarian ecotype
23. New Zealand ecotype

Figure 3.3. Principal component analysis of *L. multiflorum* and *L. perenne* populations
A. Scatterplot of the PC1 and PC2;
B. Scatterplot of the PC1 and PC3;
C. Scatterplot of the PC2 and PC4.

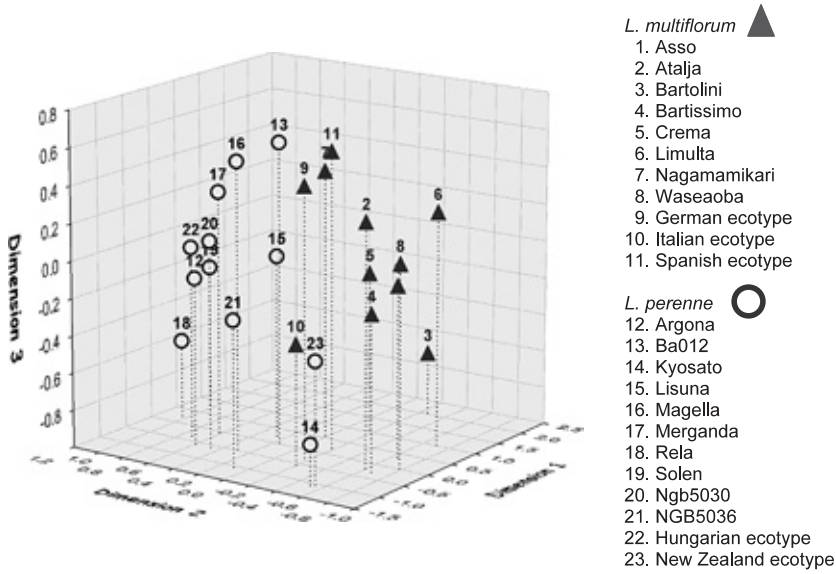


Figure 3.4. Multidimensional scaling of *L. multiforum* and *L. perenne* populations

plant flowers for the fist time or not. Response to temperature and photoperiod is closely related to geographic origin of a population and this is probably the reason that the only *L. perenne* flowered in the 1st year of cultivation originated from warm climate (New *Zeal*and). Date of ear emergence is also a subject of selection according to agronomic management and would, therefore, reflect the selective pressure during breeding programmes.

Winterhardiness is the second important species determination character. The data obtained from this study revealed that this character is also quite variable in both species. It is not surprising either that almost all Scandinavian ecotypes survived the winter without damage or low winterhardiness in *L. perenne* from New *Zeal*and. Similarly, *L. multiflorum* accessions from Italy rather did not survived the winter while the German ecotype did quite well. Although significantly greater winterhardiness has been observed in *L. perenne*, it seems that this feature strongly depends on geographical origin of a population. Very high winterhardiness of the Italian ecotype of *L. multiflorum* (80% of plants recovered after winter) appears quite surprising. However, Italian ryegrass may behave as a biennial or even short-lived perennial. In areas with reliable snow cover, it can survive for five and more years (Cosgrove et al. 1999). Unexpectedly, excellent snow cover in northern Poland could increase the chances of more southern ecotypes to survive even in comparison with areas of milder climate but often suffered from little snowing. Moreover the transgressive segregation observed in many populations suggests that winterhardiness can be increased above the average level.

On the other hand *L. perenne* is best adapted to mild-temperate climates. Plants do not stockpile well and must have good snow cover to survive the winter. This explains why some *L. perenne* cultivars were hardly recovered after the winter. It was especially true for Japan cultivar Kyosato and Polish line Ba012 (about 30% of plants). While the low winterhardiness

can also be caused by hybridization with *L. multiflorum*, this seems not plausible in this case. Inspection of their characteristics revealed them to have typical of *L. perenne* traits. They are both turf grasses that grow slowly and probably they are not able to stockpile after the cutting if it occurs too late in the autumn. The lack of winterhardiness in perennial ryegrass of turf type has been recognised. There is shortage of published reports of successful winterhardiness breeding programmes. Efforts to improve this character have been hindered by a lack of readily identifiable sources of winter-hardy germplasm (Waldron et al. 1998). That is why two *L. perenne* ecotypes from Scandinavia and Hungarian ecotype appear promising. Both of them are winter hardy in addition to not bad green and dry weight. Winterhardiness is the result of a combination of complex polygenic traits. This result in wide genetic variation as it was observed in the present studies. However, single trait based selection for improvement in any turf trait results in decreases in winterhardiness (Waldron et al. 1998). Therefore, modern turf cultivars have accumulated favorable genes for the trait under selection, but they are less winter hardy. The winterhardiness is an example of characters regarded as highly discriminant for *L. multiflorum* and *L. perenne*. Nevertheless, the high variability makes difficult its usage that again confirms the lack of sharp borders between both species.

Obviously, the classification of a highly variable species such as *L. multiflorum* and *L. perenne* really sets very difficult problems. Even though statistical differences were observed for plant weight and several generative characters, the overlapping range of variation has made the species differentiation impossible. The two species are also similar in many anatomical characters. Only minor differences were found in sclerenchyma and apex characters (Evans 1963). To complicate further the means and variation ranges of some taxonomic features (e.g., basal and flag leaf length, number of florets) are almost identical in both species. Such situation is unusual even for closely related biological species and can confirm the Bulińska-Radomska and Lester (1985) opinion that *L. multiflorum* and *L. perenne* should be re-classified as subspecies. In the absence of qualitative features, species are very often classified based on quantitative characters provided that they show a clear hiatus. Detailed analysis permits to find different morphological characters even for species that resemble each other at a first glance. By definition, cryptic species are morphologically similar but they have different gene pools that can be revealed on molecular level. When molecular fingerprints are identified and samples are properly classified based on molecular data, the morphological variation can be correlated with molecular patterns and species specific features can be determined. This relationship is mirrored elegantly in liverwort, *Pellia endiviifolia*. Three cryptic species have been identified in the Polish territory based on enzymes and semi-specific DNA markers. As soon as molecular patterns were correlated with thalus morphology, the three forms have become easily distinguishable in their natural environment by a visual appearance (Polok et al. 2005b). Similarly, in *Aneura pinquis*, morphological and ecological differences have been found following the identification of cryptic species on molecular level (Bączkiewicz et al. 2007). A considerable body of data has accumulated on high morpho-logical and ecological diversity of Scots pine (*Pinus sylvestris*). Beside its huge geographical range, plenty of diverse forms including so strange phenotype as turfosa with curved log and umbrella like crown (Polok et al. 2005a), there are no doubts that all populations belong to a single species. It can be concluded that *L. multiflorum* and *L. perenne* represent the same phenomena. The huge distribution range, a lot of ecotypes adapted to

different environments in addition to the lack of reproductive barriers - all these argue for taking them as a single entity.

The history of crops has proved that many of more dramatic differences in plant morphology are due to differences in one, or just a few genes and many have been induced by mutagenic treatment. Some of these mutants fall outside the accepted morphological limits not merely of the species, but of the genus. However, the classification keys rarely take them into account that resulted in a kind of "unnatural" classification. Crosses between these different forms readily give rise to true-breeding intermedium forms as it is observed in barley after crossing six-rowed with two-rowed cultivars or in *Lolium* as a result of hybridization between *L. multiflorum* and *L. perenne*. The latter is often classified as *Lolium* x *hybridum*. As such, it is intermediate in many traits; it is less winter hardy but higher yielding than perennial ryegrass. Regularly producing new species by crossing different forms seems absurd. In barley, for example, following a re-classification all mutants, ecotypes and cultivated forms were included in a single entity *Hordeum vulgare* (Briggs 1978). Such approach is also presented for *L. multiflorum* and *L. perenne* in the Integrated Taxonomic System of the USA (2007). This seems especially justified by human activities, which have probably caused the observed diversification of both taxa. This conclusion is supported by the distinction of *L. multiflorum* cultivars from the remaining populations. They are characterized by extremely high values for generative characters. Italian ryegrass is primary used as an annual forage crop to provide high quality grazing for dairy cattle. Therefore, it should be easy to establish, grow rapidly and produce high yields through the summer (Cosgrove 1999). The demand for improvements through breeding programmes directed to rapid grow, high quality forage and die after heading has resulted in lower winter hardiness, greater number of heading plants in the 1st year, longer spikes and greater number of spikelets in *L. multiflorum* cultivars in comparison with ecotypes. Most importantly, these predicted increases may be small but could pose a serious challenge to taxonomists. Cultivars represent the transgressive segregation and as such they fall easily outside the species mean. If selection of perennial ryegrass is directed into opposite direction i.e., to more persistent, later flowering cultivars, the separation of *L. multiflorum* and *L. perenne* may be produced artificially. This scenario seems plausible as confirmed by the lack of differences between both species at the ecotype level.

Several authors argued that breeding activities have increased the level of hybridization between *L. multiflorum* and *L. perenne* and resulted in huge amount of introgressive forms in nature (Bennett et al. 2002). This further will cause the morphological unification of both species. However, the results from this study do not support this view. Taking into account higher similarity between ecotypes of both species in comparison with cultivars, it can be speculated that differences between them have been produced recently by genetic manipulations during breeding programmes especially in *L. multiflorum*. It should be noted that in the most distinct cultivars some unusual characters were observed - thick stems, very wide, stiff and rough leaves and branched spikes. The introgression from *Festuca* species can not be therefore, excluded. Introgression is a backcrossing procedure, which allows a limited number of alien genes to be introduced from a donor species into the reconstituted genome of the recurrent species by recombination and selection. The diversity of demands on grasslands agriculture has led to increasing effort in widening the *Lolium* gene pool through the exploitation of the close relationship between *Lolium* and *Festuca*. Although less digestible

than *Lolium*, fescues are generally more persistent and can tolerate more extreme abiotic stresses (Humphreys et al. 2003). Several traits have been reported to be transferred from *F. pratensis* to *L. multiflorum*. An example is resistance to the leaf fungal disease *Puccinia coronata* Corda (crown rust). The morphology of most resistant plants resembled *L. multiflorum* completely although very occasionally single spikes were branched (Oertel and Matzk 1999). Another approach employs introgression from hexaploid *Festuca* species either from *F. arundinacea* or *F. gigantea*. Because resultant tetraploid F_1 hybrids have low fertility, the pentaploid hybrids are produced by crossing autotetraploid *L. multiflorum* and hexaploid *F. gigantea* (or *arundinacea*). After 2-3 backcrosses diploid hybrids are recovered. They are morphologically and developmentally like *L. multiflorum* but they do show some *Festuca* characters. Pentaploids hybrids are now used frequently as the starting point of introgression breeding programmes aimed at the transfer of useful characters into Italian ryegrass (Humphreys et al. 2003).

The separation of *L. multiflorum* and *L. perenne* into two species does not appear to be justified following the results of this study. The anticipated complexity of multifunctional grasslands and sustainable management practices demands the broad gene pools with options to combine complementary traits. Traditional approaches based on within-species variation are unlikely to achieve such objectives. New techniques in introgression of *Festuca* genes into *Lolium* have been more and more popular. They will inevitable lead to the diversification of Italian and perennial ryegrass cultivars and furthermore may result in species diversification. To conclude, however, the morphology alone although helpful is not enough to decide if *L. multiflorum* and *L. perenne* are separated or not. Biological species are defined by different gene pools and therefore, evidences showing whether both species have the same gene pool can confirm the above hypothesis.

3.5. CONCLUSIONS

1. The morphological analysis does not confirm the separation of *L. multiflorum* and *L. perenne* into two species. They are similar in the majority of studied characters, the range of variation overlaps and mean values are very alike.
2. The separation is possible only based on the 1st year flowering and recovery after winter although variation of both characters is present and can be connected with geographic origin and breeding activities.
3. The separate position of *L. multiflorum* cultivars results from breeding activities. Ecotypes of both species do not differ providing the evidence that differentiation of *L. multiflorum* and *L. perenne* is rather caused by human activities than evolutionary processes.

4. GENETIC DIVERSITY OF *L. MULTIFLORUM* AND *L. PERENNE*

4.1. INTRODUCTION

While the value of quantitative morphological characters may vary according to traits or environmental conditions, molecular markers are unaffected by environment providing new means to identify biological forces responsible for population genetic architectures within and between species. Although a number of techniques emerged that enable to measure genetic diversity directly at the DNA level, few other markers can match isoenzymes in the simplicity and economy of standard procedures. Comparative summaries of the allozyme data for plant taxa revealed that most plant species vary in genetic composition and that magnitudes of genetic differentiation are associated with a breeding system, reproductive mode, floral morphology and life form (Hamrick and Godt 1989). At the opposite extreme, some plants considered distinct taxonomic species show very small allozyme distances. A classic example reported in conifers e.g., *P. sylvestris* and *P. mugo* (Neet-Sarqueda 1994; Lewandowski et al. 2000) has made evident that much variation at the protein level remains hidden beyond the resolving power of conventional gel electrophoresis. Systems detecting genetic variation at the level of DNA sequence provide an alternative and sensitive method to determine genetic diversity. The availability of thousands DNA markers makes possible to scan the whole genome. Random markers are very often derived from non-coding, repetitive sequences that do not undergo the selection and where a lot of mutations can take place during species evolution. Other the past 30 years molecular markers routinely have played many key roles in description of population structure, migration, gene flow, genetic drift, natural selection, mutational divergence and many others (Karp et al. 1996; Chmiel and Polok 2005; Polok et al. 2005c). The forage grasses, which dominate many temperate agricultural and seminatural areas, have been no exception.

In the case of *Lolium* molecular markers have been primarily used to select a promising range of accessions for different breeding programmes. Among many studies more attention has been paid to identifying perennial ryegrass (Hayward and McAdam 1977; Fernando et al. 1997; Gilliland et al. 2000) and to some extent annual ryegrass (Johnson 1998) cultivars by means of isozymes. Similarly, RFLP based on ribosomal (Warpeha et al. 1998) and mitochondrial DNA (Sato et al. 1995) has been successfully used for cultivar discrimination. The diagnostic power of random amplified polymorphic DNA (RAPD) has been demonstrated both in perennial (Bolaric et al. 2005a) and annual ryegrass cultivars (Vieira et al. 2004). These experiments have shown the potential of RAPD as a rapid and reproducible method providing that high purity DNA is ensured (Bolaric et al. 2005a; Zielinski and Polok 2005). Less promising results have been obtained by restriction digestion of RAPD products. The level of polymorphism was low and useful markers were difficult to find (Sweeney and Danneberger 2000). The possibility to identify any single individual is believed to be the important advantage of more sensitive PCR methods such as AFLP and SSR. Typically both methods detect high levels of polymorphism and genetic variation within and between perennial

ryegrass gene pools (Roldan-Ruiz et al. 2001; Guthridge et al. 2001; Kubik et al. 2001). Another point is that huge genetic variation found within cultivars has hampered the usage of AFLP and SSR as a source for cultivar specific markers. A few studies have investigated the DNA diversity among *L. perenne* ecotypes (Skot et al. 2002) or compared cultivars and ecotypes (Bolaric et al. 2005b). Unfortunately, they only have concerned a single species and thus, they do not provide any insight about relationships between *L. multiflorum* and *L. perenne*. Several attempts have been made to improve identification of annual and perennial ryegrass by means of isozymes (Nakamura 1979; Payne et al. 1980) and RAPD (Zielinski et al. 1997; Zielinski and Polok 1997). But surprisingly few studies have been aimed at comparison of molecular diversity in both species.

These analyses appear to be by-products either of searchers for species specific markers or phylogenetic investigations of the whole genus with no special attention to *L. multiflorum* and *L. perenne*. Thereby they have been carried out on limited regional samples. Protein electrophoresis, for example, was used to assess the genetic variation of six *L. multiflorum* cultivars and four *L. perenne* populations from West Europe (Loos 1993b) or three *L. multiflorum* and 11 *L. perenne* populations from South-West Europe (Charmet and Balfourie 1994). Osterggaard et al. (1985) compared several *L. multiflorum* and *L. perenne* populations for five enzymes but unfortunately no population sampled in natural or seminatural sites was included. As a result, it is difficult to assess how much of the genetic variation resulted from human-mediated gene transfer. More recently, a large sample of wild and semiwild ecotypes (in total 423 accessions) has been used to array allelic diversity in comparative izoenzyme studies of four *Lolium* species, *L. remotum L. temulentum L. multiflorum* and *L. perenne* (Bennett et al. 2002). However, to maintain a realistic experimental size, the number of loci had to be limited to only five. The majority of comparative studies have been also limited by enzyme systems used. The acid phosphatase (ACP), aspartate transaminase (AAT, formerly oxaloacetate transaminase, GOT) and some dehydrogenases have been analysed the most frequently. However, the interpretation of their zymograms can be sometimes troublesome because their expression is strongly depended on the minor differences in quality of plant material. It is surprising that enzymatic systems which have proved to be very reliable in cereals (barley, wheat, rice) and other plant species have been applied in *Lolium* very rarely. Such systems include widely used esterases, peroxidases, cytosol aminopeptidase, and several others.

Far fewer studies have compared DNA diversity between *L. multiflorum* and *L. perenne*. Early examples reported that RAPD markers are more suitable for differentiation of ryegrasses than do isozymes (Zielinski et al. 1997; Zielinski and Polok 1997). More recently AFLP markers have been evaluated to distinguish genotypes, population and species of *Lolium*. Cresswell et al. (2001) compared four populations of *L. multiflorum* and three of *L. perenne* collected in Portugal with three AFLP primer combinations. An important feature was the remarkable high dimensionality of the data at all levels of variation. Plants from the same population tended to cluster together with minimal overlap between populations and species. One important drawback to these studies includes arbitrary classification of all the samples with variable number of florets as *Lolium* x *hybridum*. Other noteworthy studies using eight AFLP primer combinations have confirmed the high genetic similarity between Italian and perennial ryegrass (Polok et al. 2006). They were in-

deed concentrated on the whole genus and only single samples of *L. multiflorum* and *L. perenne* were assessed.

The examples cited above indicate that direct comparisons of Italian and perennial ryegrasses have been avoided. Instead, considerable attention has been devoted to comparison of *L. perenne* with *L. rigidum* (Balfourier et al. 1998) or even with members of the other grass genera (Warren et al. 1998; Kolliker et al. 1999). Restriction analysis of chloroplast genome has been used in order to explain the present distribution area of natural populations of *L. perenne* and *L. rigidum* (Balfourier et al. 2000). It is so strange that *L. rigidum* has hardly economic importance otherwise being a rare and threaten weed. *L. multiflorum*, on the other hand, is one of most important temperate forage species that continues to cause problems during breeding due to high morphological similarity to *L. perenne*. One likely explanation for the relative lack of data regarding *L. multiflorum* is that the results have been too confusing and disagreed with the present taxonomic classification.

The aim of the present study was therefore, to review the differentiation of *L. multiflorum* and *L. perenne* by means of several molecular marker systems including those not used previously. To determine how much of the genetic variation has resulted from human mediated gene flow and what remains of natural pattern of variation, both cultivars and wild ecotypes were included in the analysis. To maximize effectiveness by combining high resolution with broad coverage of individuals, isoenzymes with several DNA methods were applied. It is still expensive and time-consuming to obtain DNA information from multiple Mendelian nuclear loci among numerous individuals. Therefore, isozyme electrophoresis was applied at the beginning. It remains one of the best techniques available for studies of population structure also because of codominance. Restriction and PCR analysis of chloroplast and mitochondrial DNA is valuable method for estimation of migration routes and up to our knowledge no comparison between cpDNA and mtDNA of *L. multiflorum* and *L. perenne* has been made. Among random DNA markers, RAPDs were chosen due to simplicity and efficiency in species determination. So called semi-specific markers are similar to RAPDs in that they employ a single primer to amplify DNA. However, they are based on the consensus sequences for the intron splice junctions (ISJ). The junctions to exons are highly conserved sequences. Introns are generally subjected to weak selective pressure and therefore, they are usually highly variable in sequence length. These properties would appear to make the ISJs ideal targets for the identification of polymorphism in PCR products (Weining and Landridge 1991). This type of markers has never been used in ryegrasses but proved to be highly effective in discrimination of pea and barley mutants (K. Polok, unpublished data). Moreover, transposon-based profiling (SSAP) was optimized and for the first time applied for estimation of genetic diversity in *L. multiflorum* and *L. perenne*. Polymorphism resulted from transposon insertion is thought to be higher than revealed by AFLP and especially useful for intra-specific comparisons (Syed et al. 2005). Transposons are able not only to increase significantly the host genome size but can also mutate host genes. Therefore, they are likely to be a major contributor to the generation of genetic diversity in plants (Kumar et al. 1997). To gain an overall picture of this diversity, both DNA and retrotransposon based SSAP was used.

4.2. MATERIAL AND METHODS

Twenty five populations of *L. multiflorum* and *L. perenne* representing cultivars and wild ecotypes and two cultivars of *L. hybridum* were analysed by means of isoenzymes and from twelve to twenty by different DNA markers. The list of populations and the methods of material development are presented in Annex 13.1. On average 30 plants from each population were used in isozyme analysis and 10 plants per a population in DNA analyses. For each plant about 5-10 g of newly emerged leaves were harvested four weeks after the autumn cut (i.e., in early October). Then they were packed in labelled bags, placed on ice, transported to the laboratory and frozen in 30ºC for enzyme and DNA isolation. Since there was no loss of enzyme activity in frozen plants in comparison with fresh ones, only frozen material was used in further studies. The enzyme analysis and DNA isolation were carried 1-4 months after the leaf harvesting.

4.2.1. Isoenzyme analysis

Isozymes were assayed by horizontal starch gel electrophoresis in Lithium borate/Tris-citrate buffer system. Polymorphism was measured at seven enzyme systems: esterases (EST), fluorescent esterases (EST-flu), aspartate transaminase (AAT), cytosol aminopeptidase (CAP), NAD-depended malate dehydrogenase (MDH), peroxidases (PER) and superoxide dismutase (SOD). The methods, buffer recipes and staining procedures were taken from Zielinski (1987) with own modifications. All procedures and enzyme systems used are described in Annex 13.3.

Interpretation of zymograms

Due to the lack of knowledge about genetics of all studied isozymes but AAT, all loci were identified by comparison with a model plant - *Hordeum vulgare*. The relatively high similarity between genomes of Poaceae makes possible to use a single species as a model for the analysis of the others. Therefore, with a broad knowledge about genetics and structure of the majority of enzymes, relatively close similarity to *Lolium* and the same chromosome number (2n=14), barley is a very suitable model in analysis of isozymes in ryegrasses. Later on the manner of inheritance of all studied isozymes was confirmed in crossing experiments (Chapter 6).

In the present work the standardised numbering system proposed by Kahler and Allard (1970) for barley was adopted. Enzyme systems were referred to by upper case letters according to standard names (e.g., CAP, EST etc.). Specific loci were noted using enzyme system identification but with lower case letters except for the first one (e.g., *Cap1*, *Est1*). For each locus the bands were labelled according to their distance in mm from the origin. Subsequent bands were numbered for example, 20, 30, 35 etc. Homozygous genotypes were thus, labelled 20/20 for example and heterozygous 20/30 for example. Specific alleles were denoted after hyphen, *Cap1-20, Est1-25*. This system allows both for easy comparison between own and literature data and numbering of new alleles. The similar system was also adopted for AAT, GPI and ACP by Hayward et al. (1995).

4.2.2. DNA analysis

Total DNA was isolated from individual plants according to the modified CTAB procedure (Annex 13.4). Five types of molecular markers were used in the studies. Sequences of all primers used are given in Annex 13.5. Chloroplast DNA was analysed by restriction digestion (*Dra*II, *EcoR*I and *Hae*III) of the non-coding region *psbC-trnS* in chloroplast genome (Annex 13.6). Mitochondrial DNA was analysed by amplification polymorphism of intron between B and C exons of *nad1* gene (Annex 13.7). Total DNA was analysed by random amplified polymorphic DNA - RAPD (Annex 13.8), intron splice junction polymorphism - ISJ (Annex 13.9) and transposon based sequence specific amplification polymorphism - SSAP (Annex 13.12). After initial screening of 30 RAPD (kit A from Operon and ten designed primers) and 12 ISJ primers, in total nine RAPD primers (KP01-KP07, OPA02, OPA03) and four ISJ primers (ISJ1-ISJ4) were chosen. The SSAP transposon specific primer was designed to amplify the *Lolium* DNA transposon, *Tpo1* belonging to the CACTA superfamily (Langdon et al. 2003). Moreover two primers, LTR1 and LTR2 designed on the basis of consensus *Ty1-copia* LTR sequences (RNAseH-LTR junction) were applied. The LTR1 was based on *Lolium multiflorum* and *Oryza sativa* sequences while LTR2 on *Saccharum officinarum* and *Triticum aestivum* sequences. These three primers were used in combinations with three AFLP primers, two *Mse*I specific primers (Mse-CT, Mse-ACA) and a single *Pst*I specific primer (Pst-AT). In the case of RAPD, ISJ and SSAP all bands that could be reliable read were treated as single dominant loci and scored present (1) or absent (0) across all genotypes. Identity of the majority of bands with the same mobility in *L. multiflorum* and *L. perenne* and their nuclear inheritance were previously confirmed in genetic mapping studies (Chapter 6). Thus, RAPD, ISJ and SSAP can be treated as nuclear dominant markers.

Data analysis

Allele frequency in each population, the average number of alleles over all loci (A), effective number of alleles (N_e), percent of polymorphic loci (P), gene diversity (heterozygosity) at each locus (h) and the average gene diversity in a population over all loci (H) were calculated by the POPGENE version 1.32 (Yeh and Yang 1999) according to Nei (1987).

Population genetic statistics were also calculated for ecotypes, cultivars and for each species that were treated as populations divided into a number of subpopulations. The following statistics were calculated on a population basis: the mean number of alleles per locus ($\bar{A}$), the mean effective number of alleles ($\bar{N}_e$), average gene diversity at a locus ($\bar{h}$), the total gene diversity (H_T) decomposed into the gene diversities within subpopulations (H_S) and between subpopulations (D_{ST}), the coefficient of gene differentiation, G_{ST} that is identical to Wright F_{ST}. The gene diversity (H_T, H_S, D_{ST} and G_{ST}) were first calculated for each locus separately and then averaged. Moreover the fixation index in a group of populations (F_{IT}) and the average in each population (F_{IS}) was calculated. The deviation of F_{IT} and F_{IS} from zero was calculated by t-test. If the number of alleles is large, the distribution of parameters is approximately normal, and the ordinary statistical tests may be used. Therefore, the statistical significance of population genetic parameters corresponding to the different groups (ecotypes, cultivars) or species was evaluated by one-way analysis of variance with LSD test using STATISTICA 7.1 software. The overview of this statistics is presented in Annex 13.15.

The frequencies of enzymatic alleles found were used as variables in the data matrix in multivariate analysis. The frequencies were transformed using the arcsin to reduce skewness. Principal component analysis was performed to establish which alleles explained the most of the observed variation and furthermore to construct a PC plot of populations. The PCA was calculated using a correlation matrix.

Nei's genetic identities or normalized identities of genes (1972; 1987) were calculated on the basis of allele frequency data between pairs of populations, between ecotypes and cultivars as well as between species with a POPGENE 1.32. The resultant matrices were used in the cluster analysis and multidimensional scaling of STATISTICA 7.1. The Euclidean distance and unweighted pair-group method using arithmetic averages (UPGMA) were used to form the dendrograms.

4.3. RESULTS

4.3.1. Polymorphism of *L. multiflorum* and *L. perenne*

Enzyme polymorphism

Fifteen enzymatic loci with 42 alleles were identified in the joined analysis of twenty five populations of *L. multiflorum* and *L. perenne* representing cultivars and wild ecotypes. Twelve loci were polymorphic (80% of loci) with average 1.96 and 1.81 alleles per a locus in *L. multiflorum* and *L. perenne*, respectively. The level of polymorphism and the number of alleles per a locus did not differ in both species. These values were also very alike in cultivars and ecotypes (Table 4.1). The analysis of allele frequencies did not reveal any significant differences between species. The same alleles with similar frequencies were present in cultivars and ecotypes (Table 4.2). No single allele was diagnostic neither to species nor ecotypes and cultivars. Four alleles observed only in *L. perenne* (*Est2-33, Est4-null, Aat3-null, Per1-45*) could not be regarded as diagnostics because their frequency was very low. Similarly, two alleles, *Est3-20* and *Per2-23* could not be treated as specific to *L. multiflorum*. The lack of differences between *L. multiflorum* and *L. perenne* on enzymatic level was confirmed by Principal Component Analysis based on allele frequencies. Five principal components explained 65% of enzymatic variation. The scatterplot diagrams on the first three principal components show that Italian ryegrass populations are dispersed within perennial ryegrass populations (Figure 4.1A-B). The separate position is only occupied by one cultivar of *L. hybridum*, Gosia.

Table 4.1. Genetic variation in *L. multiflorum* and *L. perenne*

Method	N° of loci/ bands	Genetic variation	*L. multiflorum*	*L. perenne*	*L. multiflorum*		*L. perenne*	
					cultivars	ecotypes	cultivars	ecotypes
Enzymes	15	L	12	12	12	12	12	12
		P	80.00	80.00	80.00	80.00	80.00	80.00
		Ā	1.96	1.81	1.91	1.63	1.89	1.99
		N_e	1.53	1.65	1.60	1.39	1.59	1.69
cpDNA	5	L	2	3	1	2	3	3
		P	40.00	60.00	20.00	40.00	60.00	60.00
		Ā	1.40	1.60	1.40	1.20	1.60	1.60
		N_e	1.16	1.48	1.08	1.24	1.48	1.48
mtDNA	11	L	8	7	6	6	5	6
		P	72.73	63.64	54.55	54.55	45.45	54.55
		Ā	1.72	1.63	1.55	1.55	1.45	1.55
		N_e	1.43	1.38	1.34	1.39	1.36	1.39
RAPD	64	L	48	49	47	38	39	40
		P	75.00	76.56	73.43	59.58	60.94	62.50
		Ā	1.75	1.70	1.79	1.71	1.68	1.72
		N_e	1.44	1.44	1.42	1.46	1.44	1.43
ISJ	55	L	51	53	51	29	51	49
		P	92.72	96.36	92.73	52.73	92.72	89.09
		Ā	**1.73**^{b}	**1.91**^{a}	**1.92**^{a}	**1.53**^{b}	**1.93**^{a}	**1.89**^{a}
		N_e	1.40	1.51	1.42	1.37	1.50	1.53
SSAP *Tpo1*	229	L	188	162	149	156	134	121
		P	82.10	70.74	65.07	68.12	58.52	52.84
		Ā	**1.82**^{a}	**1.71**^{b}	**1.68**^{a}	**1.65**^{ab}	**1.59**^{ab}	**1.53**^{b}
		N_e	1.43	1.44	1.36	1.40	1.39	1.34
SSAP *Lolcopia1*	160	L	133	95	92	100	63	67
		P	83.12	59.38	57.50	62.50	39.38	41.88
		Ā	**1.83**^{a}	**1.59**^{b}	**1.58**^{ab}	**1.63**^{a}	**1.39**^{b}	**1.42**^{ab}
		N_e	**1.41**^{a}	**1.32**^{b}	**1.30**^{ab}	**1.36**^{a}	**1.23**^{b}	**1.28**^{ab}
SSAP *Lolcopia2*	251	L	191	181	151	155	128	145
		P	76.10	72.11	60.16	61.75	51.00	57.77
		Ā	1.76	1.72	1.60	1.62	1.51	1.58
		N_e	1.48	1.51	1.38	1.41	1.35	1.40

L – number of polymorphic loci/bands, P – percent of polymorphic loci/bands, Ā – mean number of alleles (bands) per a locus (site), N_e – mean number of effective alleles per a locus,
^{a}different letters mean significant differences at P=0.05

Table 4.2. Allelic frequencies summarized for 15 enzyme loci in *L. multiflorum* and *L. perenne* and in ecotypes and cultivars of both species

Locus	Allele	*L. multiflorum*	*L. perenne*	*L. multiflorum*		*L. perenne*	
				cultivars	ecotypes	cultivars	ecotypes
Est1	*Est1-15*	0.014	0.003	0.025	0.007	0.000	0.007
	Est1-12	0.657	0.644	0.695	0.636	0.697	0.560
	Est1-07	0.265	0.238	0.280	0.256	0.262	0.201
	Est1-null	0.064	0.115	0.000	0.101	0.041	0.232
Est2	*Est2-40*	0.288	0.472	0.130	0.379	0.503	0.423
	Est2-35	0.712	0.520	0.870	0.621	0.497	0.556
	Est2-33	0.000	0.008	0.000	0.000	0.000	0.021
Est3	*Est3-28*	0.124	0.172	0.200	0.057	0.261	0.084
	Est3-22	0.601	0.617	0.374	0.814	0.633	0.600
	Est3-20	0.019	0.000	0.033	0.000	0.000	0.000
	Est3-null	0.256	0.211	0.393	0.129	0.106	0.316
Est4	*Est4-49*	0.835	0.794	0.917	0.787	0.856	0.696
	Est4-45	0.165	0.139	0.083	0.213	0.115	0.175
	Est4-null	0.000	0.067	0.000	0.000	0.029	0.129
Est-flu3	*Est-flu3-47*	0.644	0.696	0.420	0.700	0.176	0.655
	Est-flu3-45	0.356	0.304	0.580	0.300	0.284	0.345
Aat1	*Aat1-43*	1.000	1.000	1.000	1.000	1.000	1.000
Aat2	*Aat2-40*	0.120	0.143	0.100	0.134	0.144	0.132
	Aat2-36	0.215	0.225	0.105	0.225	0.199	0.250
	Aat2-32	0.581	0.430	0.757	0.563	0.411	0.489
	Aat2-null	0.084	0.202	0.038	0.078	0.246	0.129
Aat3	*Aat3-15*	0.052	0.162	0.045	0.083	0.196	0.109
	Aat3-12	0.400	0.253	0.500	0.321	0.246	0.264
	Aat3-07	0.548	0.577	0.455	0.596	0.546	0.623
	Aat3-null	0.000	0.008	0.000	0.000	0.012	0.004
Cap1	*Cap1-47*	1.000	1.000	1.000	1.000	1.000	1.000
Cap2	*Cap2-42*	1.000	1.000	1.000	1.000	1.000	1.000
Mdh1	*Mdh1-45*	0.017	0.017	0.000	0.033	0.000	0.033
	Mdh1-42	0.983	0.983	1.000	0.967	1.000	0.967
Mdh2	*Mdh2-37*	0.000	0.050	0.000	0.000	0.067	0.033
	Mdh2-34	0.850	0.817	0.767	0.933	0.833	0.800
	Mdh2-32	0.150	0.133	0.233	0.067	0.100	0.167
Per1	*Per1-45*	0.000	0.006	0.000	0.000	0.009	0.000
	Per1-38	0.150	0.025	0.225	0.107	0.009	0.050
	Per1-35	0.635	0.830	0.609	0.650	0.769	0.926
	Per1-null	0.215	0.139	0.167	0.243	0.213	0.024
Per2	*Per2-32*	0.551	0.634	0.550	0.553	0.633	0.634
	Per2-26	0.370	0.275	0.450	0.289	0.367	0.183
	Perx2-23	0.036	0.000	0.000	0.053	0.000	0.000
	Per2-null	0.053	0.091	0.000	0.105	0.000	0.183
Sod1	*Sod1-60*	0.786	0.643	0.920	0.753	0.884	0.163
	Sod1-54	0.214	0.357	0.080	0.248	0.116	0.837

Allelic frequencies in *L. multiflorum* and *L. perenne* did not differ statistically by LSD test at P=0.05. No significant differences were observed between cultivars and ecotypes of both species neither.

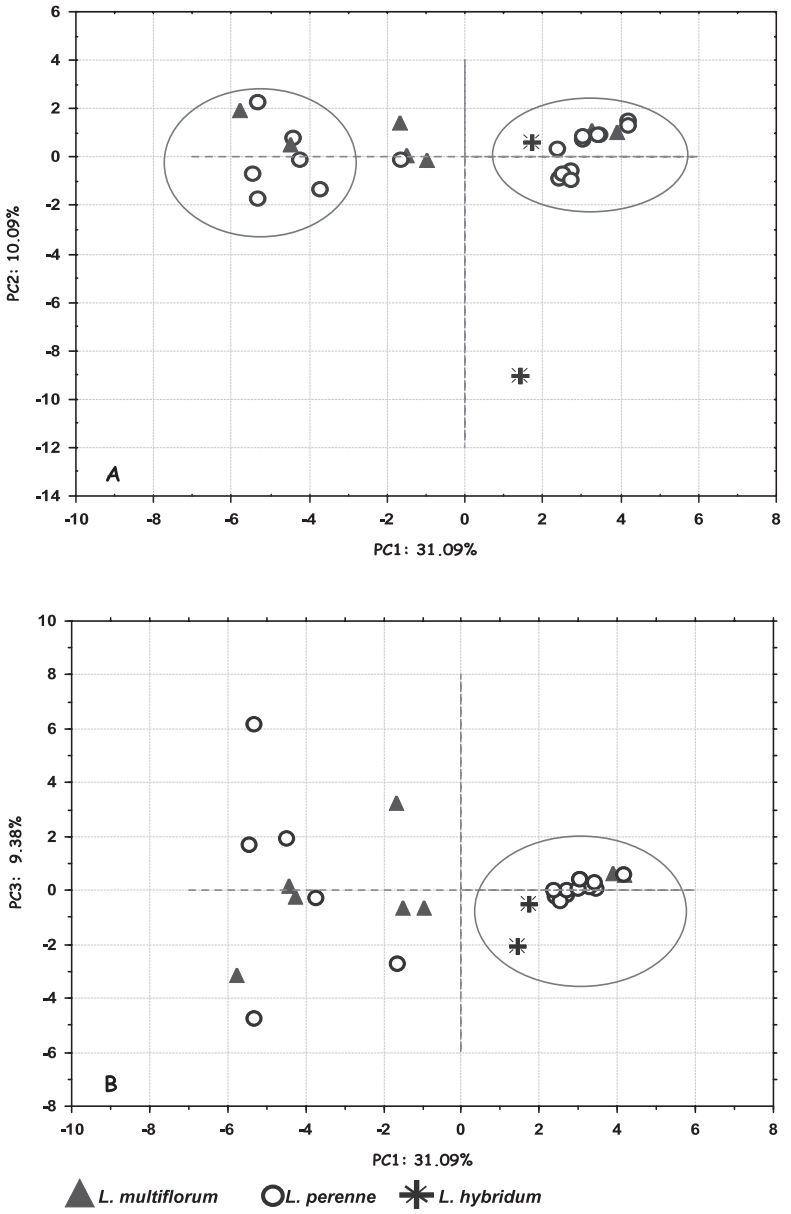


Figure 4.1. Scatterplot of 27 *Lolium* populations on the first three principal components based on enzymatic allele frequencies

Organelle DNA polymorphism

Five restriction fragments were identified in *psbC-trnS* after *Hae*III digestion. The other two enzymes, *Dra*II and *Eco*RI did not cut this chloroplast DNA fragment. A total of 11 PCR products were observed after amplification of *Lolium* DNA with primers specific to the intron between B and C exons in *nad1* gene. Mitochondrial origin of revealed fragments was proved in F_1 hybrid of a cross between *L. multiflorum* (Bartolini) and *L. perenne* (ecotype from New *Zea*land) and 20 F_2 individuals. The female haplotype was distinguished by the presence of six bands in comparison with ten in the male one. The F_1 hybrid possessed all six bands typical of female parent and none of the male parent. Moreover, the female haplotype was present in all 20 F_2 individuals.

The level of polymorphism was relatively low for organelle DNA in comparison with enzymes and the nuclear DNA markers used (Table 4.1). Of the five fragments scored from cpDNA, only two (40%) were polymorphic in *L. multiflorum* and three (60%) in *L. perenne*. This resulted in as few as six haplotypes identified (Figure 4.2). The most frequent were haplotypes nº1 and nº2, which were shared equally by both species (Table 4.3). The rest haplotypes were rare, and nearly all were characteristic to *L. perenne*. Only the nº4 was typical of *L. multiflorum* ecotype, Variamo (Table 4.4).

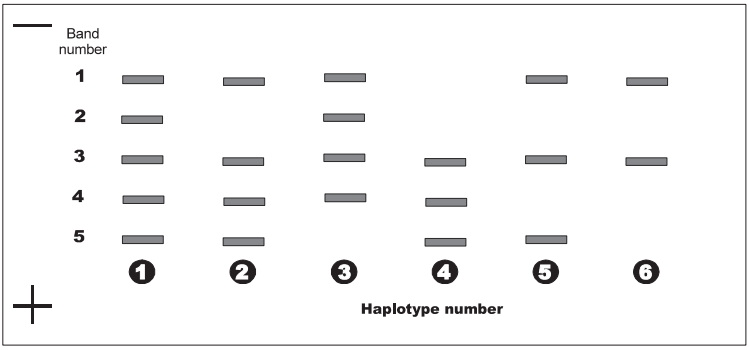


Figure 4.2. Description of the six cpDNA haplotypes identified in *L. multiflorum* and *L. perenne*

A slightly higher proportion of polymorphic bands was observed in mitochondrial DNA of both species (72 and 63%). The values in cultivars and ecotypes were quite similar (Table 4.1). In contrast to cpDNA more than doubled number of haplotypes was produced (Figure 4.3). Only three of them were present both in *L. multiflorum* and *L. perenne*. The haplotype nº3 was observed with relatively high frequency in both species (0.250 and 0.334). It was also equally frequent in cultivars and ecotypes (Table 4.3). In contrast, the other two shared haplotypes (nº4 and nº11) were rare and specific to a population (Table 4.4). Each of them was harboured by a single population per a species. The majority of mitochondrial haplotypes (76%) were rare and specific not only to a species but also to a population. *L. multiflorum* harboured the unique haplotypes nº5, 8, 9, 10 and 13 whereas *L. perenne* - nº1, 2, 6, 7 and 12. If we considered populations studied, the most distinct was ecotype from the Tatras because it harboured both cpDNA and mtDNA haplotypes not present anywhere else.

Table 4.3. Frequencies of chloroplast and mitochondrial haplotypes in *L. multiflorum* and *L. perenne* and in ecotypes and cultivars of both species

DNA	Haplotype N°	*L. multiflorum*	*L. perenne*	*L. multiflorum*		*L. perenne*	
				cultivars	ecotypes	cultivars	ecotypes
cpDNA	1	0.250	0.250	0.333	0.167	0.333	0.167
	2	0.667	0.333	0.833	0.500	0.333	0.332
	3	0.000	0.083	0.000	0.000	0.000	0.167
	4	0.083	0.000	0.000	0.167	0.000	0.000
	5	0.000	0.084	0.000	0.000	0.000	0.167
	6	0.000	0.250	0.000	0.000	0.334	0.167
mtDNA	1	0.000	0.083	0.000	0.000	0.167	0.000
	2	0.000	0.083	0.000	0.000	0.000	0.167
	3	0.250	0.334	0.167	0.333	0.333	0.333
	4	0.083	0.083	0.000	0.166	0.167	0.000
	5	0.167	0.000	0.167	0.167	0.000	0.000
	6	0.000	0.167	0.000	0.000	0.000	0.333
	7	0.000	0.083	0.000	0.000	0.166	0.000
	8	0.084	0.000	0.000	0.167	0.000	0.000
	9	0.083	0.000	0.167	0.000	0.000	0.000
	10	0.083	0.000	0.167	0.000	0.000	0.000
	11	0.083	0.084	0.000	0.167	0.000	0.167
	12	0.000	0.083	0.000	0.000	0.167	0.000
	13	0.167	0.000	0.332	0.000	0.000	0.000

Haplotype frequencies in *L. multiflorum* and *L. perenne* did not differ statistically by LSD test at P=0.05. No significant differences were observed between cultivars and ecotypes of both species neither.

RAPD and ISJ polymorphism

A total of 119 polymorphic bands were detected among eight populations of *L. multiflorum* and 12 populations of *L. perenne*. In both species the ISJ markers revealed higher polymorphism than RAPD (Table 4.1) measured as the percentage of polymorphic loci. Similarly, the number of scorable bands per a primer was almost twice greater for ISJs (13.8) than for RAPDs (7.1). Italian and perennial ryegrass appeared to have the similar level of polymorphism as estimated by RAPD (75 and 77%, respectively) and ISJ markers (93 and 96%). They did not differ in the average number of RAPD alleles over all loci (A). In opposite, this parameter was lower in *L. multiflorum* than in *L. perenne* when estimated from ISJ markers. This probably resulted from the very low polymorphism observed in ecotypes of *L. multiflorum*. Otherwise, cultivars of Italian ryegrass had the polymorphism comparable to both cultivars and ecotypes of perennial ryegrass.

In general, *L. multiflorum* and *L. perenne* shared the same bands with similar frequencies. None of identified bands was a species specific i.e., present in all individuals of one species but absent in another. It was only possible to find two “semi-diagnostic alleles” i.e., alleles that are generally present in one species and rarely found in the other. Both alleles were generated by the RAPD primer, OPA02. They were observed with significantly higher frequency in *L. multiflorum* in comparison with *L. perenne*, namely 0.423 and 0.242 for OPA02-4 and 0.399 and 0.163 for OPA02-4. Interestingly, the latter was present in all individuals of *L. multiflorum* cultivars but it was very rare (frequency equal to 0.069) in ecotypes of this species.

Table 4.4. Occurrence of chloroplast and mitochondrial haplotypes in cultivars and ecotypes of *L. multiflorum* and *L. perenne*

DNA	Haplotype N°	*L. multiflorum*						*L. perenne*					
		cultivars			ecotypes			cultivars			ecotypes		
		Atalja	Bartissimo	Bartolini	Barball	Guliamo	Variamo	Lisuna	Magella	Portstewart	NGB4264	NGB10809	Tatras
cpDNA	1		■			■	■	■			■		
	2	■	■	■	■	■			■		■	■	
	3												▨
	4						▤						
	5												▨
	6									▨		▨	
mtDNA	1								▨				
	2											▨	
	3		■				■	■	■		■		
	4				■					■			
	5			▤		▤							
	6												▨
	7									▨			
	8					▤							
	9			▤									
	10		▤										
	11				■							■	
	12							▨					
	13	▤											

▤ haplotypes present only in *L. multiflorum*
▨ haplotypes present only in *L. perenne*

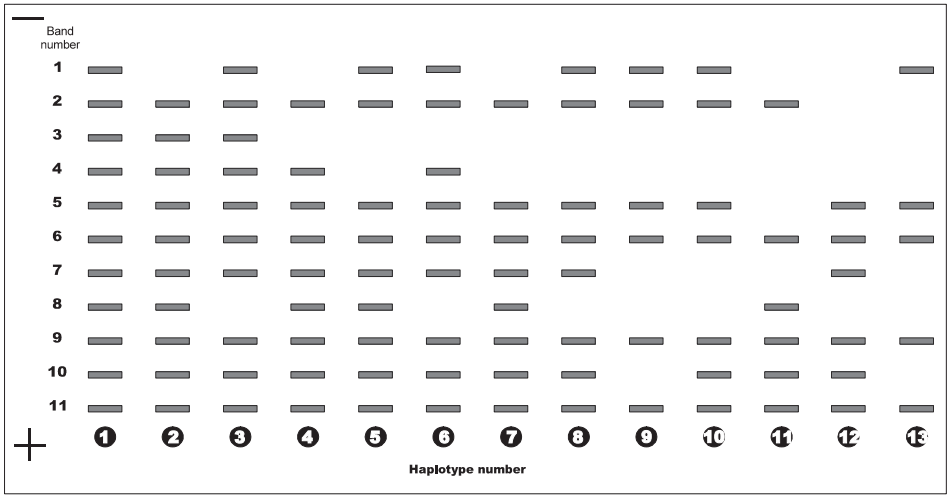


Figure 4.3. Description of the 13 mtDNA haplotypes identified in *L. multiflorum* and *L. perenne*

SSAP polymorphism

The primer complementary to *L. perenne* CACTA transposon, *Tpo1* produced a great number of bands in *L. multiflorum* and *L. perenne* thus, confirming the high abundance of *Tpo1* in these two ryegrasses. Similarly, each of the two LTR primers complementary to *Ty1-copia* consensus sequences produced bands on SSAP gel for each species, showing that each transposon is present within them (Figure 4.4A). Therefore, the transposons revealed by LTR1 and LTR2 were further assigned as *Lolcopia1* and *Lolcopia2*, respectively.

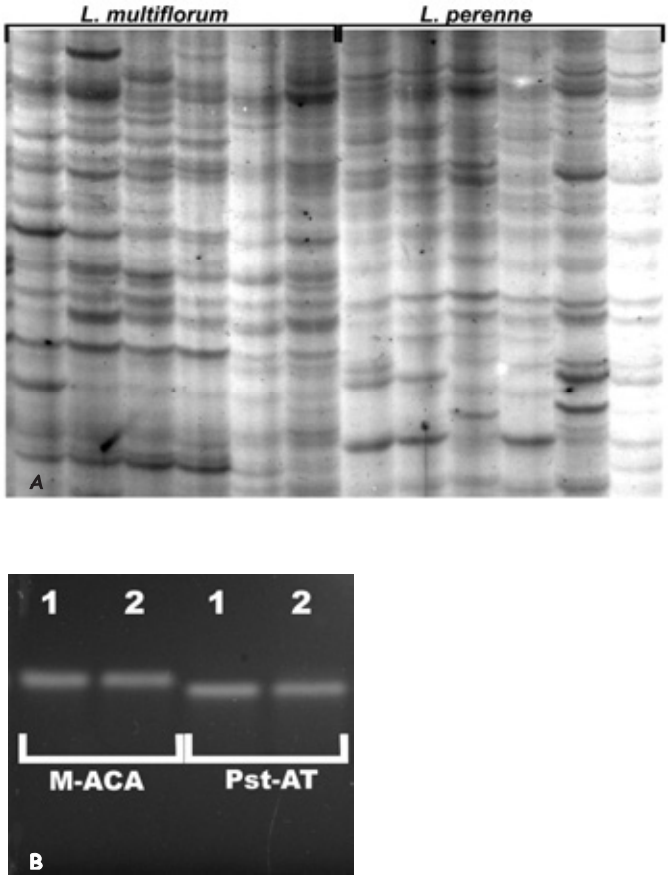


Figure 4.4. Insertional polymorphism of *Lolcopia2* transposon in *L. multiflorum* and *L. perenne*
A. SSAP analysis with Mse-ACA primer
B. Reamplification of bands obtained from SSAP with Mse-ACA and Pst-AT

All transposon sequences studied were highly polymorphic among *L. multiflorum* and *L. perenne* (Table 4.1), indicating that they have been transposing within these species in the recent past. The overall polymorphism level for each transposon was the same for *Tpo1* and *Lolcopia1* i.e., 93% and 88% for *Lolcopia2*. The proportion of polymorphic sites and the

mean number of bands per a site were higher in *L. multiflorum* than in *L. perenne*, especially for *Tpo1* and *Lolcopia1*. These two parameters did not differ between species in the case of the third transposon, *Lolcopia2*.

In Italian ryegrass, the results showed the comparable insertional polymorphism level for *Tpo1* (82%) and *Lolcopia1* (83%) and lower for *Lolcopia2* (76%). By contrary, in *L. perenne* this last transposon showed almost equal level of polymorphism to *Tpo1* (72 and 70%, respectively) while the lowest value was given by *Lolcopia1* (59%). The differences in percentage may indicate that each transposon has a characteristic level of transposition within Italian and perennial ryegrass. Inspection of cultivars and ecotypes of both species confirmed higher number of insertion sites in *L. multiflorum*. In both species higher level of polymorphism was observed in ecotypes. This is in opposite to RAPD and ISJ results but not surprising as cultivars have been generally derived from a narrow gene pool. However, this relationship was not so obvious when taking into account the mean number of bands per a site because they did not differ between ecotypes and cultivars within a species.

There was a great amount of band sharing between both species (Figure 4.4A). No differences were observed in several bands of the same mobility in both species after the elution from the polyacrylamide gel and their re-amplification (Figure 4.4B). Despite the high resolution of SSAP and a great number of bands identified, species specific markers could be scarcely found and they constituted only 1.9% (12 bands) of all identified bands. Two of employed transposons had unique insertion sites for each species. The DNA transposon, *Tpo1*, revealed one site in each species (Figure 4.5) and the retrotransposon *Lolcopia1*, five sites in *L. multiflorum* and three in *L. perenne* (Figure 4.6). The other retrotransposon, *Lolcopia2* was found in three specific sites only in *L. perenne* (Figure 4.7). Moreover, there were significant differences in transposon frequency between species for about 3% of insertion sites.

4.3.2. Genetic diversity of *L. multiflorum* and *L. perenne*

The value of genetic variability in *L. multiflorum* and *L. perenne* was almost identical and the majority of Nei's genetic parameters did not differ between them (Table 4.5). Both species had relatively large total gene diversity (H_T) that ranged from 0.253 to 0.344 in Italian ryegrass and from 0.202 to 0.340 in perennial ryegrass. About 40-60% of this variation was distributed between populations as estimated from G_{ST}. Also the D_{ST} value informing about the among-population gene diversity was usually higher than H_s informing about the within-population gene diversity. The significant deviation from zero of the fixation index (F_{IT}) was observed in both species for the majority of marker systems applied. The differences between *L. multiflorum* and *L. perenne* were displayed only in few cases. The former had higher transposon diversity (higher H_T, H_S and G_{ST} for *Tpo1* and *Lolcopia1*) while in the latter splice junctions and cpDNA were more diverged (higher values of D_{ST} and G_{ST}).

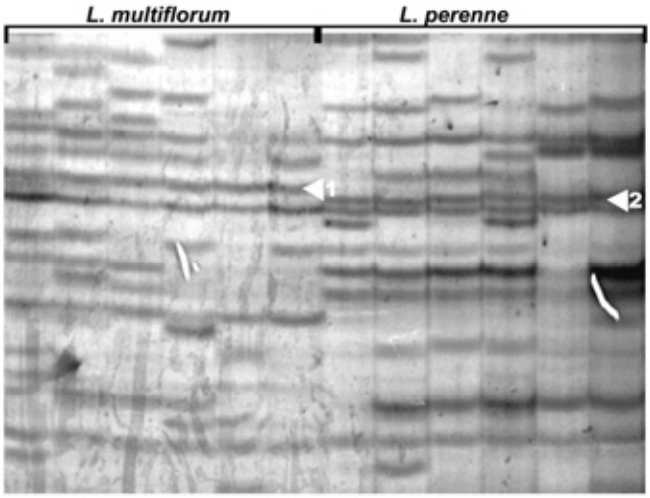


Figure 4.5. Insertional polymorphism of *Tpo1* transposon in *L. multiflorum* and *L. perenne* revealed by SSAP analysis with Mse–CT primer
(Arrows indicated bands specific to 1. *L. multiflorum*, 2. *L. perenne)*

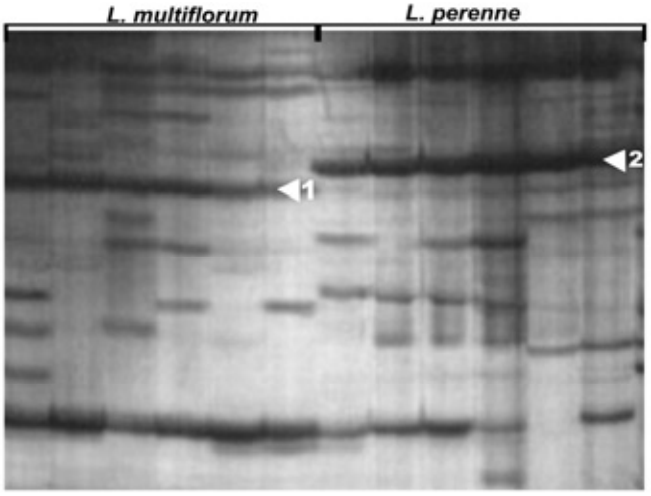


Figure 4.6. Insertional polymorphism of *Lolcopia1* transposon in *L. multiflorum* and *L. perenne* revealed by SSAP analysis with Pst–AT primer
(Arrows indicated bands specific to 1. *L. multiflorum*, 2. *L. perenne*)

It can be assumed that these minor differences are related with microevolutionary processes from one side, and with breeding activities from another. For example, the lower diversity of *Lolcopia1* in both cultivars and ecotypes of *L. perenne* may indicate that this is a species specific feature responsible for ongoing ryegrass diversification. Moreover, the lower divergence of DNA transposon, *Tpo1* in *L. perenne* ecotypes in comparison with

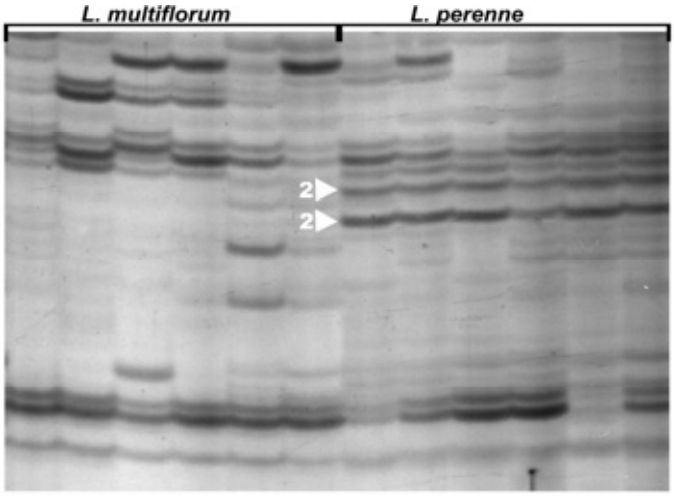


Figure 4.7. Insertional polymorphism of *Lolcopia2* transposon in *L. multiflorum* and *L. perenne* revealed by SSAP analysis with Pst-AT primer
(Arrows indicated bands specific to 1. *L. multiflorum*, 2. *L. perenne*)

L. multiflorum ecotypes again justifies their recent origin. The major drawback of this explanation is the same level of gene diversity displayed by cultivars. The third transposon *Lolcopia2* seems not to play an important role in ryegrass speciation as can be concluded from similar values of Nei's diversity parameters for ecotypes. It is plausible that the lower divergence of *Lolcopia2* in perennial ryegrass cultivars resulted from breeding activities. The cultivars that have to be uniform are derived from a limited number of individuals. This was confirmed by extremely low cpDNA diversity in *L. multiflorum* cultivars for which gene diversity indices were about threefold lower than in ecotypes and sevenfold lower than in *L. perenne*.

The marker type had some effect on the values of population genetic parameters in Italian and perennial ryegrass. The largest discrepancy was observed between H_S and D_{ST} estimated from enzymes and DNA markers. Firstly, allozyme revealed the two-threefold higher the within-population gene diversity then did DNA markers. This difference was rather expected since the latter type of markers can only produce two alleles in each locus, and therefore, the maximum gene diversity is 0.5 whereas multiallelic markers such as isoenzymes can produce values up to one. Nevertheless, the H_S obtained by the analysis of transposon insertion sites (SSAP) was surprisingly low that can suggest the fixation of specific transposon pools in each population. As a result most of genetic variation was distributed between populations. Secondly, in the case of enzymes, the within-population gene diversity (H_S) was higher than the between-population variation (D_{ST}), which is common for cross-breeding species. This relationshipwas inverted for all DNA markers that again support the thesis about the narrow genetic background of each population.

Table 4.5. Nei's genetic diversity statistics summarized for *L. multiflorum* and *L. perenne*

Method	Parameter	*L. multiflorum*	*L. perenne*	*L. multiflorum*		*L. perenne*	
				cultivars	ecotypes	cultivars	ecotypes
Enzymes	H_T	0.344	0.340	0.344	0.344	0.340	0.340
	H_S	0.269	0.288	0.301	0.213	0.273	0.298
	D_{ST}	0.145	0.083	0.134	0.165	0.086	0.082
	G_{ST} (F_{ST})	0.421	0.245	0.389	0.478	0.253	0.240
	F_{IT}	0.219	0.152	0.125	0.382	0.196	0.124
	F_{IS}	0.001	0.001	0.001	0.002	0.002	0.001
cpDNA	H_T	0.092	0.270	**0.035**^{b}	**0.123**^{ab}	**0.266**^{a}	**0.262**^{a}
	H_S	0.055	0.083	**0.028**^{b}	**0.083**^{ab}	**0.001**^{b}	**0.166**^{a}
	D_{ST}	**0.036**^{b}	**0.187**^{a}	**0.007**^{b}	**0.041**^{b}	**0.265**^{a}	**0.096**^{ab}
	G_{ST} (F_{ST})	0.168	0.414	**0.043**^{b}	**0.148**^{b}	**0.600**^{a}	**0.203**^{ab}
	F_{IT}	0.275	*0.629**	0.048	0.259	*1.000**	0.237
	F_{IS}	-0.205	-0.212	0.123	-0.205	*1.000**	-0.205
mtDNA	H_T	0.253	0.241	0.162	0.235	0.213	0.228
	H_S	0.063	0.094	0.075	0.050	0.087	0.100
	D_{ST}	0.191	0.147	0.086	0.185	0.125	0.128
	G_{ST} (F_{ST})	0.560	0.393	0.226	0.425	0.268	0.294
	F_{IT}	*0.636**	*0.528**	0.437	0.603	0.500	0.470
	F_{IS}	-0.460	-0.211	-0.209	-0.867	-0.208	-0.213
RAPD	H_T	0.267	0.272	0.252	0.260	0.249	0.253
	H_S	0.124	0.096	0.120	0.130	0.106	0.086
	D_{ST}	0.143	0.177	0.147	0.137	0.167	0.187
	G_{ST} (F_{ST})	0.536	0.649	0.549	0.514	0.612	0.686
	F_{IT}	*0.558**	*0.696**	*0.522**	*0.501**	*0.576**	*0.662**
	F_{IS}	0.279	0.045	-0.003	0.002	0.004	0.006
ISJ	H_T	0.280	0.315	0.257	0.206	0.306	0.309
	H_S	0.172	0.100	0.163	0.186	0.088	0.111
	D_{ST}	**0.108**^{b}	**0.215**^{a}	**0.117**^{b}	**0.094**^{b}	**0.227**^{a}	**0.204**^{ab}
	G_{ST} (F_{ST})	**0.403**^{b}	**0.683**^{a}	**0.417**^{ab}	**0.335**^{b}	**0.720**^{a}	**0.647**^{ab}
	F_{IT}	***0.386****^{b}	***0.683****^{a}	***0.352****^{b}	**0.283**^{b}	***0.711****^{a}	***0.639****^{ab}
	F_{IS}	0.009	0.016	-0.002	-0.018	-0.038	-0.030
SSAP *Tpo I*	H_T	0.278	0.264	**0.246**^{a}	**0.261**^{a}	**0.235**^{a}	**0.199**^{b}
	H_S	**0.089**^{a}	**0.068**^{b}	**0.096**^{a}	**0.081**^{ab}	**0.077**^{b}	**0.058**^{c}
	D_{ST}	0.189	0.196	**0.149**^{a}	**0.179**^{a}	**0.159**^{a}	**0.141**^{b}
	G_{ST} (F_{ST})	0.500	0.508	**0.356**^{a}	**0.420**^{a}	**0.366**^{a}	**0.335**^{b}
	F_{IT}	*0.679**	*0.744**	*0.606**	*0.689**	*0.674**	*0.705**
	F_{IS}	0.000	0.005	0.000	-0.010	0.004	-0.012
SSAP *Lolcopia1*	H_T	**0.261**^{a}	**0.202**^{b}	**0.199**^{ab}	**0.222**^{a}	**0.155**^{c}	**0.158**^{bc}
	H_S	**0.079**^{a}	**0.048**^{b}	**0.085**^{a}	**0.074**^{ab}	**0.040**^{c}	**0.056**^{bc}
	D_{ST}	0.182	0.155	**0.115**^{ab}	**0.147**^{a}	**0.115**^{ab}	**0.102**^{b}
	G_{ST} (F_{ST})	**0.501**^{a}	**0.416**^{b}	**0.281**^{ab}	**0.360**^{a}	**0.267**^{b}	**0.246**^{b}
	F_{IT}	*0.696**	*0.763**	*0.576**	*0.665**	*0.744**	*0.646**
	F_{IS}	-0.004	0.004	0.008	-0.005	0.008	0.000
SSAP *Lolcopia2*	H_T	0.282	0.280	**0.228**^{ab}	**0.237**^{a}	**0.195**^{b}	**0.238**^{a}
	H_S	0.079	0.079	0.075	0.083	0.076	0.083
	D_{ST}	0.202	0.201	**0.152**^{a}	**0.154**^{a}	**0.119**^{b}	**0.154**^{a}
	G_{ST} (F_{ST})	0.511	0.499	**0.361**^{a}	**0.364**^{a}	**0.286**^{b}	**0.354**^{a}
	F_{IT}	*0.719**	*0.715**	*0.670**	*0.650**	*0.610**	*0.650**
	F_{IS}	-0.002	-0.008	-0.004	0.000	0.000	-0.004

[a]different letters mean significant differences at P=0.05, *significant deviation from 0 at P=0.05 (calculated only for F_{IT} and F_{IS})

4.3.3. Genetic similarity of *L. multiflorum* and *L. perenne*

Estimates of Nei's genetic identities between *L. multiflorum* and *L. perenne* were very high (Table 4.6), well within the range of values normally associated with conspecific populations. The highest value was obtained for enzymatic loci (0.986). The analysis of random and semi-random nuclear DNA markers revealed only slightly lower values ranging from 0.925 for RAPDs to 0.976 for ISJs. It is not a surprise that the highest degree of differentiation was observed for insertional polymorphism but still the values were much higher than for distinct species (0.887-40.896).

Table 4.6. Nei's genetic identity between *L. multiflorum* and *L. perenne* based on different molecular markers

	Enzymes	cpDNA	mtDNA	RAPD	ISJ	*Tpo1*	*Lolcopia1*	*Lolcopia2*
I	0.986	0.957	0.961	0.925	0.976	0.895	0.887	0.896

The similarities between ecotypes and cultivars within each species were high (0.821-0.999) although the degree of differentiation did not differ from that for interspecific comparisons (0.783-0.999). The only exception was the higher similarity of transposons within a species. The Nei's coefficients for within species comparisons ranged from 0.848 to 0.950 whereas genetic similarities between species ranged from 0.783 to 0.871. Surprisingly, cultivars of *L. multiflorum* were quite different from ecotypes while estimating from RAPDs (I=0.821) and ISJs (I=0.867). Such a relationship was not observed in *L. perenne* thus, suggesting different breeding strategies in both species (Table 4.7).

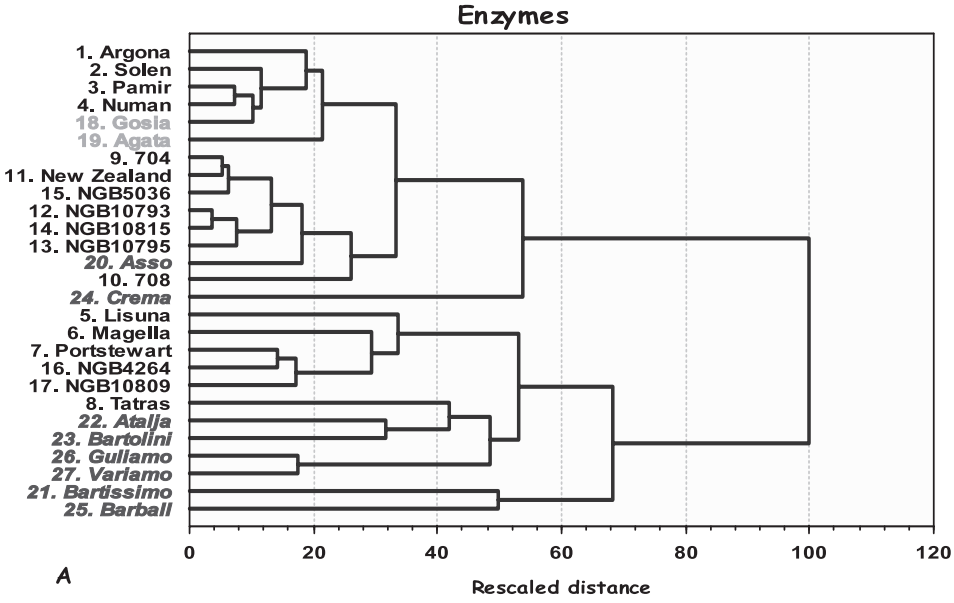
Table 4.7. Nei's genetic identity between cultivars and ecotypes of *L. multiflorum* and *L. perenne* based on different molecular markers

Marker type	Within species comparisons		Between species comparisons			
	L. multiflorum Cultivars vs. Ecotypes	*L. perenne* Cultivars vs. Ecotypes	*L. multiflorum* Cultivars vs. *L. perenne* Cultivars	*L. multiflorum* Ecotypes vs. *L. perenne* Ecotypes	*L. multiflorum* Cultivars vs. *L. perenne* Ecotypes	*L. multiflorum* Ecotypes vs. *L. perenne* Cultivars
Enzymes	0.957	0.930	0.948	0.952	0.985	0.896
cpDNA	0.999	0.985	0.967	0.963	0.967	0.963
mtDNA	0.907	0.995	0.872	0.999	0.867	0.999
RAPD	0.821	0.954	0.852	0.917	0.872	0.914
ISJ	0.867	0.980	0.950	0.949	0.933	0.910
Tpo1	0.950	0.921	0.843	0.858	0.856	0.871
Lolcopia1	0.901	0.915	0.813	0.838	0.797	0.869
Lolcopia2	0.902	0.848	0.783	0.861	0.841	0.823

When we instead turn to interspecific comparisons, ecotypes were more similar than cultivars, again indicating the influence of breeding activities on species differentiation. In some cases, the Nei's coefficients for ecotypes were even higher than for within-species comparisons (Table 4.7). For example mtDNA of Italian and perennial ryegrass was more similar (0.999) than mtDNA of cultivars and ecotypes within *L. multiflorum* (0.907). What is more, the cultivars of *L. multiflorum* appeared to be closer related with *L. perenne* ecotypes

than with ecotypes of their parent species while justifying from enzymes, RAPDs and ISJs. On opposite, *L. perenne* cultivars remained to be the most similar to *L. perenne* ecotypes.

Cluster analysis and multidimensional scaling based on genetic similarities showed clearly the ability of the majority of marker systems used to discriminate all populations (Figure 4.8-4.15). The only exception was chloroplast DNA in *L. multiflorum* which enabled to identify two groups with the same cpDNA haplotype in all populations within a group (Figure 4.9A). These difficulties in separation of populations were also demonstrated in the multidimensional scaling scatterplot. The distinct points were formed only by four *L. perenne* populations (Figure 4.9B). In the case of the majority of marker system used, neither dendrograms nor scatterplots separated populations in accordance to their taxonomic position. In general, ecotypes of *L. multiflorum* tended to group together with *L. perenne* while cultivars of this species formed a distinct cluster. The highest resolution was obtained by insertional polymorphism. In the UPGMA dendrograms two clusters could be identified, the first with all populations of *L. multiflorum* but Barball, and the second with *L. perenne* populations and Barball (Figure 4.13-4.15). The multidimensional scaling emphasised the difference between Italian and perennial ryegrass populations in the case of *Tpo1* and *Lolcopia1*. On opposite, all populations were scattered through the whole space in the *Lolcopia2* plot irrespectively of the rotation applied (Figure 4.15B). Even the lower discrimination between species was obtained by RAPDs and ISJs. The grouping based on RAPDs (Figure 4.11A) generated a separate cluster of the three *L. multiflorum* ecotypes (Barball, Guliamo, Variamo), *L. perenne* cultivar (Lisuna) and *L. perenne* ecotype from the Tatras. This then merged with *L. perenne* ecotypes from Scandinavia (NGB4264, NGB10809). A strong overlap was also obtained when a multidimensional scaling was applied (Figure 4.11B). When the dendrogram was plotted based on ISJs two larger clusters can be observed, both with accessions of *L. multiflorum* and *L. perenne* (Figure 4.12). Similarly, enzymes grouped all populations into two clusters with accessions from both species in each cluster although the majority of *L. multiflorum* populations formed a minor separate cluster (Figure 4.8). However this is not so obvious in the scatterplot. Interestingly, *L. hybridum* was rather clustered with *L. perenne*. The data from organelle DNA contrasted with the other marker system in that the majority of populations were continuously distributed in the UPGMA dendrograms and scatterplots (Figure 4.9-4.10).



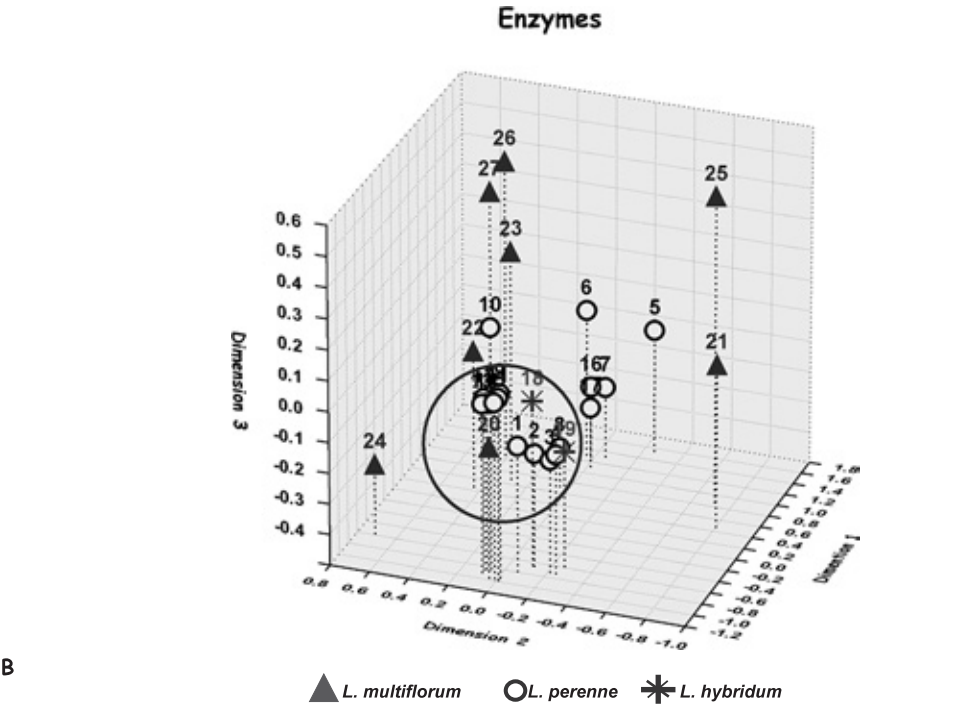
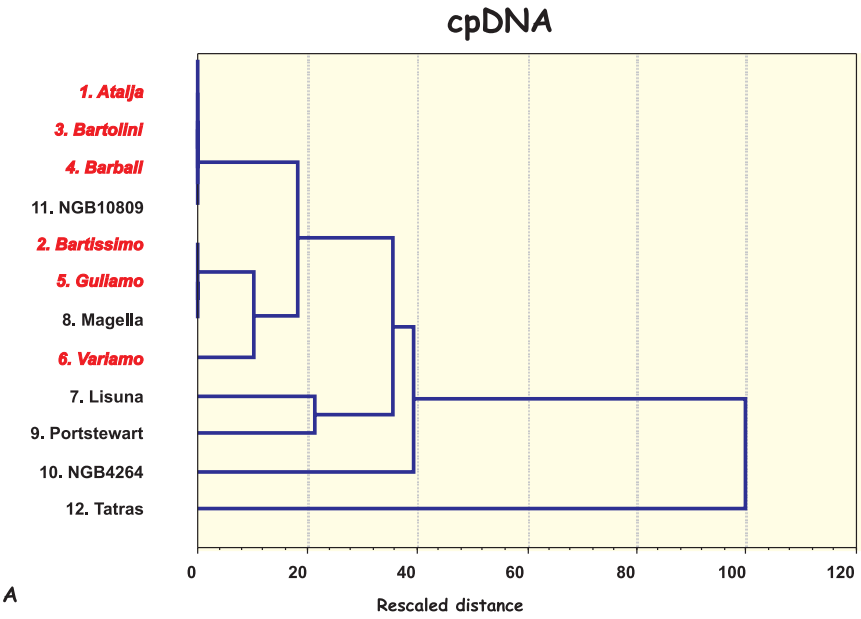
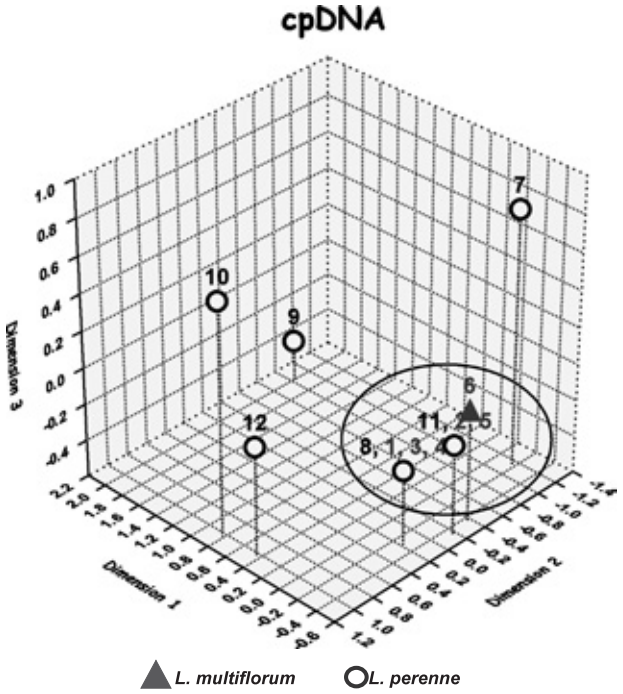


Figure 4.8. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on isoenzymes

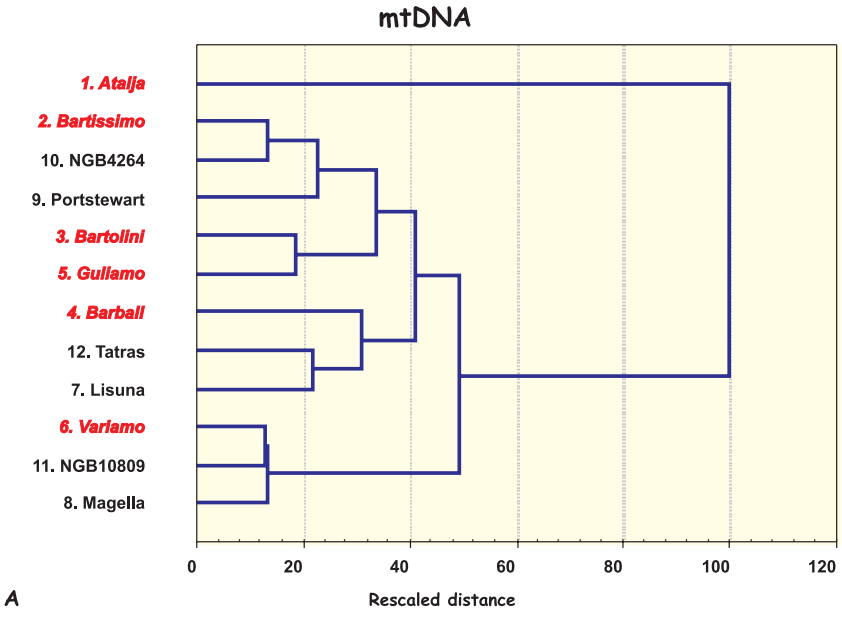


A



B

Figure 4.9. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on cpDNA



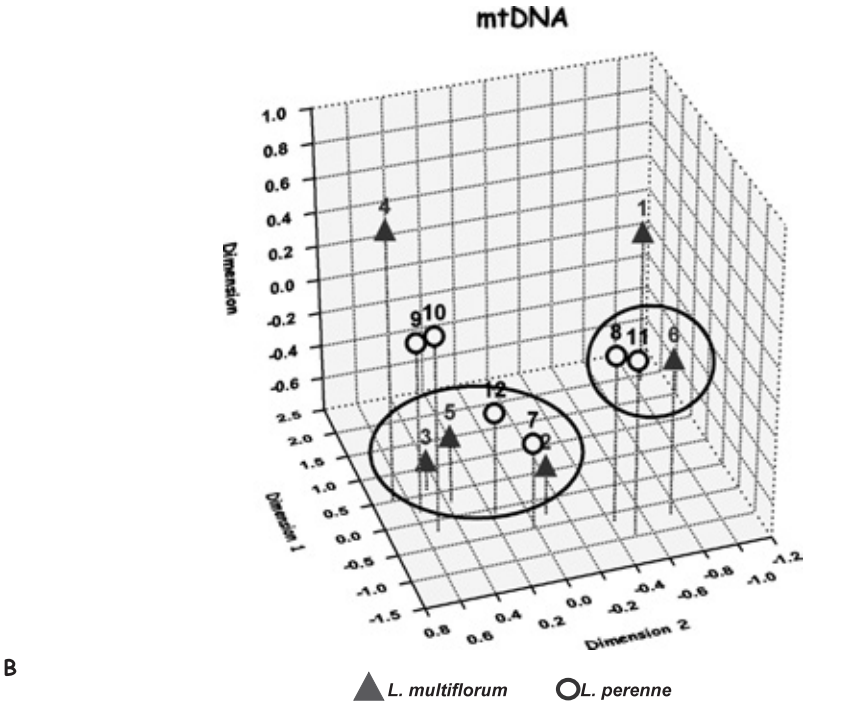
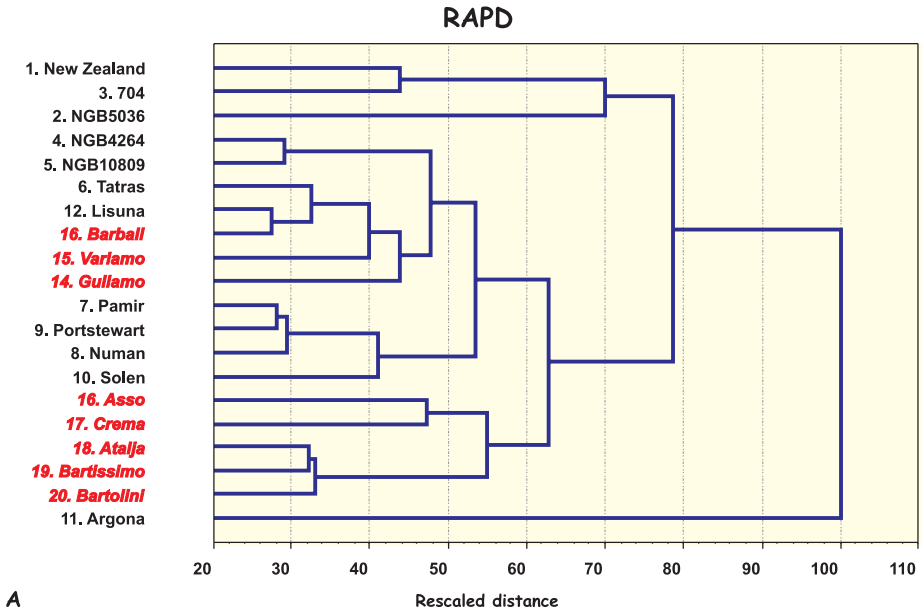
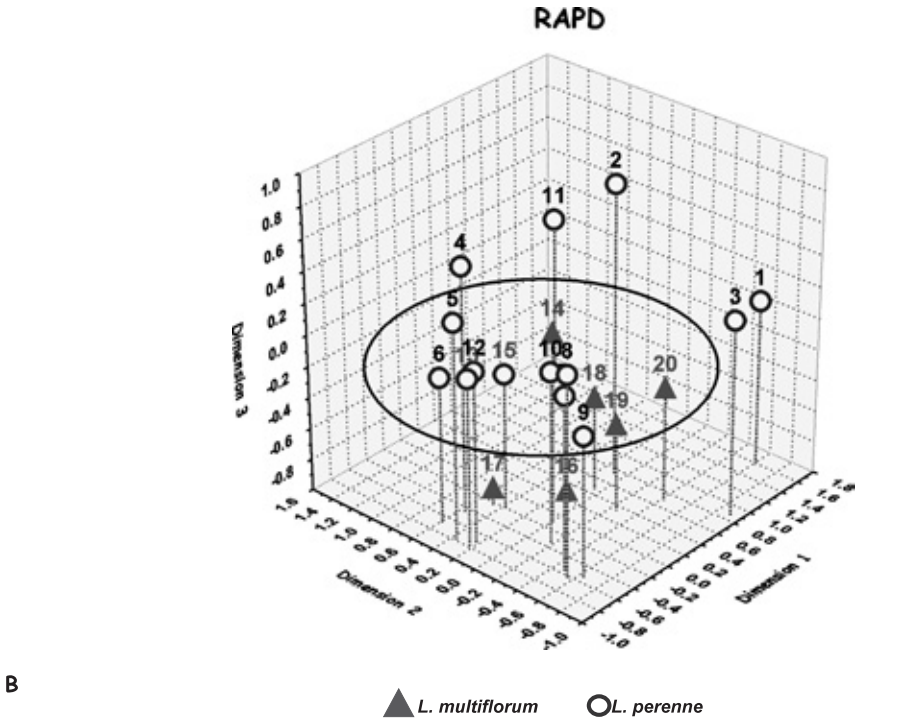


Figure 4.10. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on mtDNA



A



B

Figure 4.11. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on RAPD

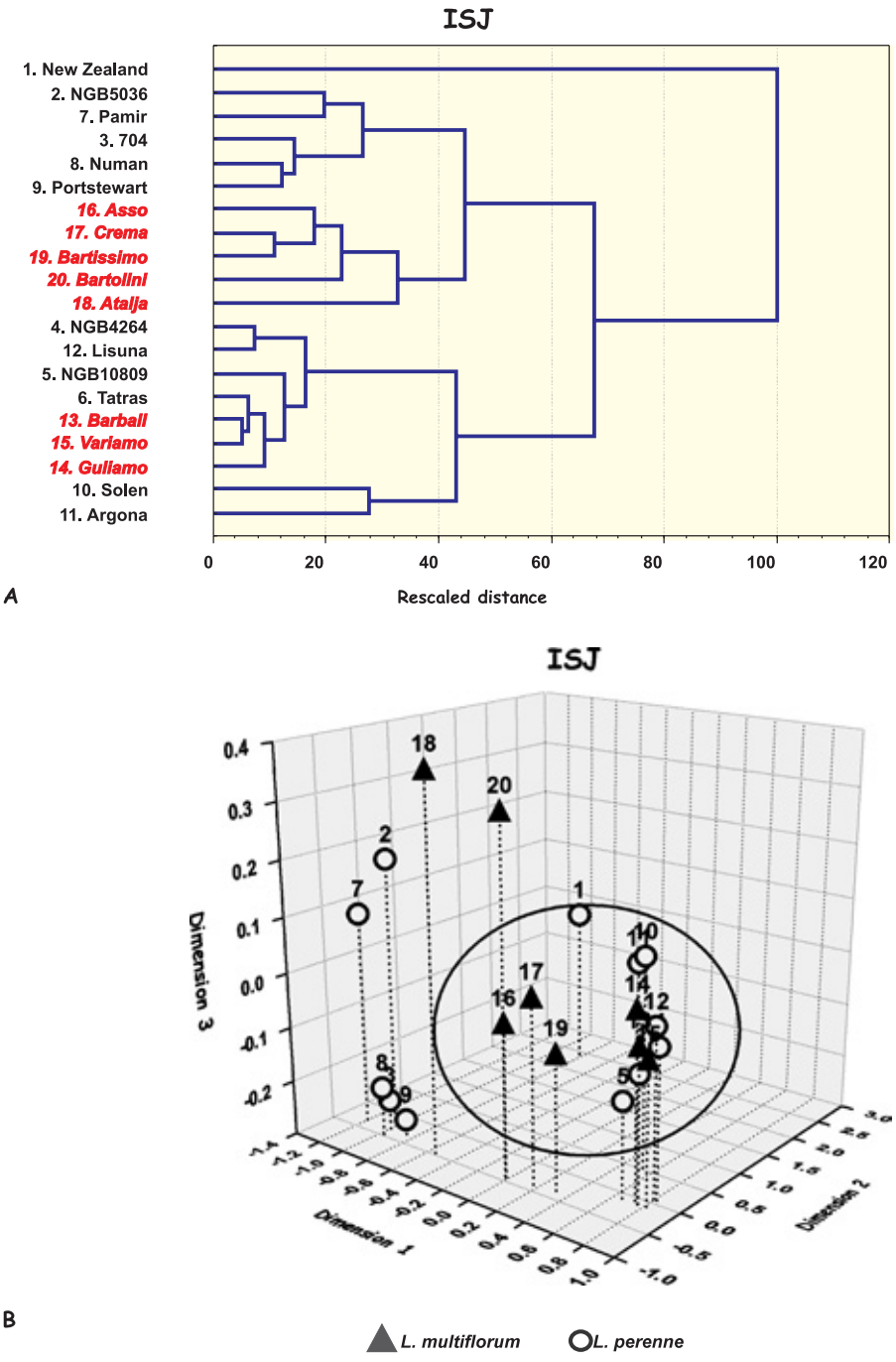
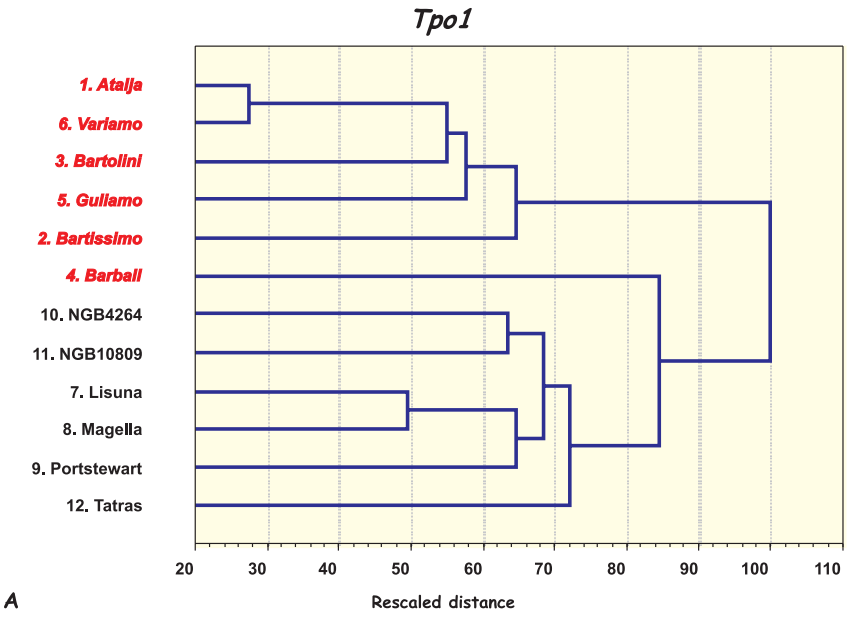
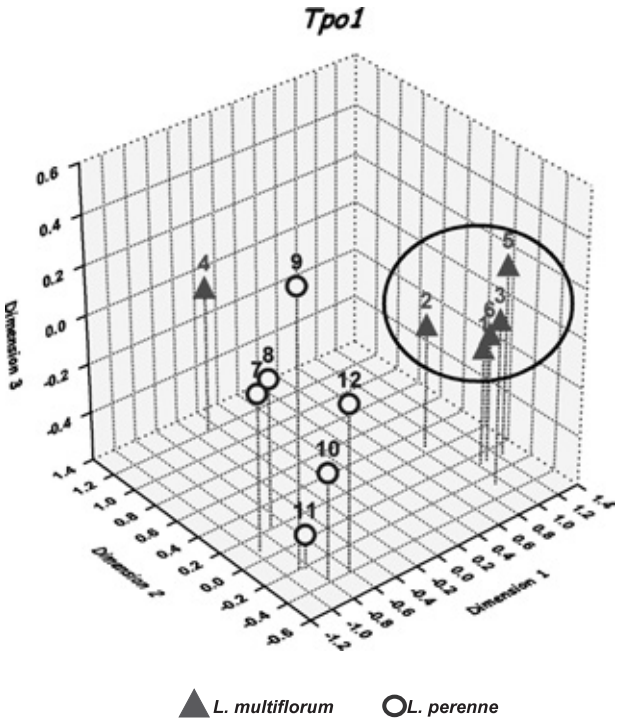


Figure 4.12. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on ISJ

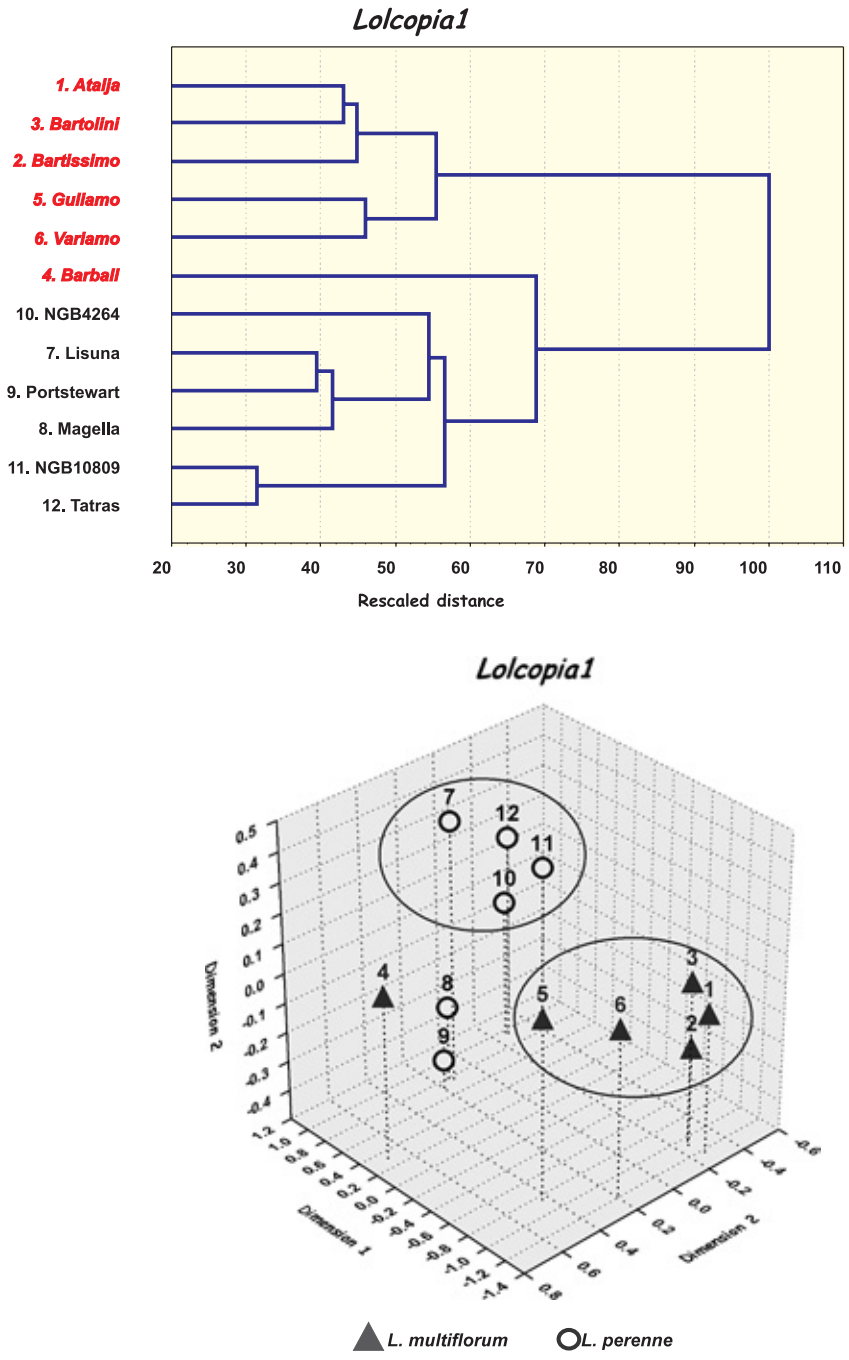


A



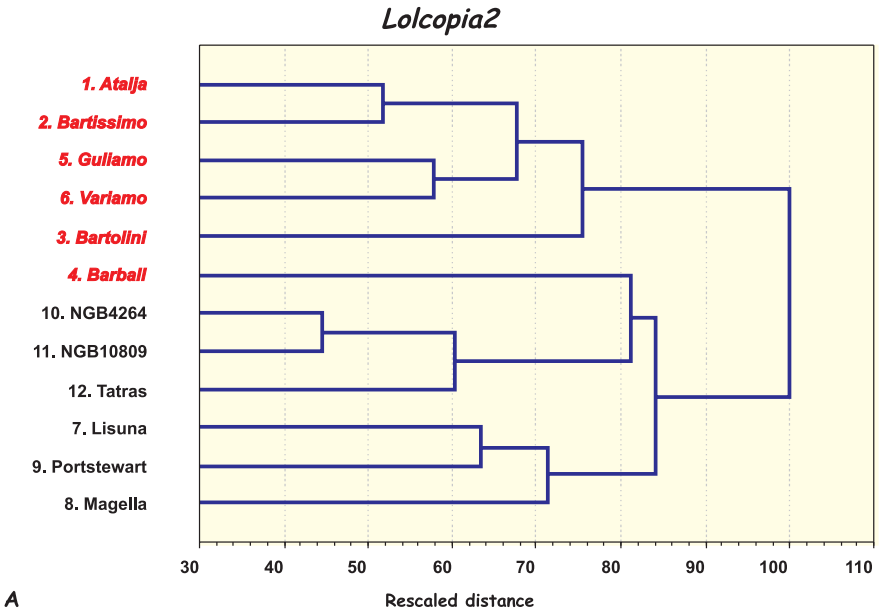
B

Figure 4.13. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on *Tpo1* DNA transposon



B

Figure 4.14. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on *Lolcopia1* retrotransposon



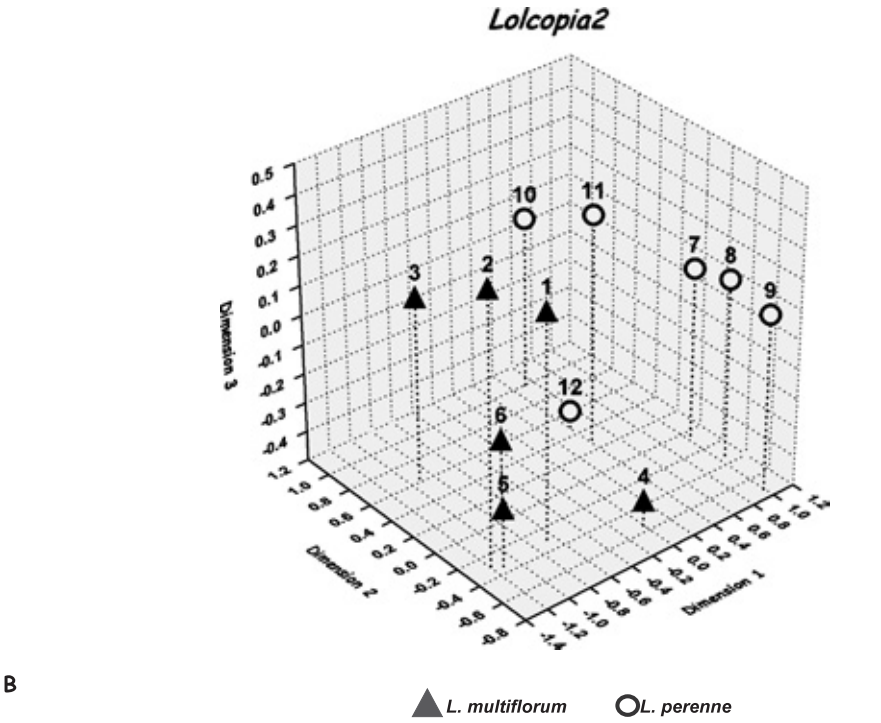


Figure 4.15. UPGMA grouping (A) and multidimensional scaling (B) of *L. multiflorum* and *L. perenne* based on *Lolcopia2* retrotransposon

4.4. DISCUSSION

4.4.1. Genetic variation in *L. multiflorum* and *L. perenne*

The genetic polymorphisms observed in populations are the products of various evolutionary forces in their long history. Thus, the extent of genetic variability is characteristic for each species but ecological and life history factors have an effect on plant population genetic structure. The average proportion of polymorphic enzymatic loci (P) in plants is about 46% (Futuyma 1986), the mean number of alleles per locus (A) is 1.53 and the mean gene diversity (H) - 0.113 (Hamrick and Godt 1989). However, it has been well documented that much higher genetic diversity exists in out-crossing species. In *Trifolium* for instance, allogamous species have three times more polymorphic loci in comparison with autogamous ones (73% and 27%, respectively) and the number of enzymatic alleles is almost doubled (2.1 and 1.2). Likewise, the total (H_T=0.343) and within-population gene diversities (H_S=0.249) are significantly higher in the former (Bulinska-Radomska 2005). By contrast, the proportion of polymorphic loci in self-pollinated species tends to be very low even thought high-throughout technologies have been applied. Only 2.3% of polymorphic AFLP bands have been found in annual, inbreeding grass *Bromus tectorum* (Ramakrishnan et al. 2004) but no monomorphic AFLP band has been observed in out-breeding *Pseudoroegneria spicata* (Larson et al. 2004). Similar results have been obtained with RAPD or ISJs markers. As many as 83% of polymorphic loci have been detected in *Pinus cembra* from the restricted area of the Tatras, despite the fact that this population is very small (Chmiel and Polok 2005). The genus *Lolium* grouping both autogamous and allogamous species is not an exception. As it can be concluded from mainly enzymatic studies the genetic structure of inbreeding and cross-breeding species is completely different (Table 4.8). Very low electrophoretic variation has been found in self-pollinating species concluding from the average 6% of polymorphic loci, 1.08 alleles per a locus and gene diversity equal 0.029. Accordingly, the majority of populations are completely fixed for a single allelic variant (Loos 1993b). However, none of enzymatic variants present in inbreeding species is unique, all have been also found in cross-breeders (Polok 2005). By contrary, allogamous species are highly variable with 76% to 91% of polymorphic loci, 2.13-3.25 alleles per a locus and the average gene diversity 0.277-0.405 (Table 4.8). *L. rigidum* has proved to be the most variable species. Therefore, a high degree of enzymatic variation recorded in *L. multiflorum* and *L. perenne* in the present study and reflected by 80% of polymorphic loci, 1.81-1.96 alleles per a locus and the total gene diversity about 0.340 falls well within the range typical of allogamous, wind-pollinated *Lolium* species.

Analyses of extensive data compilations have demonstrated that allozyme-derived population genetic parameters are comparable across studies. But they have suffered from a relatively low number of loci and might provide misrepresentative estimates of genetic variation because the availability of histochemical stains has been a deciding criterion for the inclusion in electrophoretic surveys. In the present research 15 enzymatic loci were identified of which 12 were polymorphic (80%) in *L. multiflorum* and *L. perenne*. With virtually unlimited amount of potential markers, DNA based methods provide much more efficient tool to disclose genetic diversity in any population and species. The present results have shown that genomes of *L. multiflorum* and *L. perenne* harbour a wealth of variation on DNA level. The resolution

Table 4.8. Genetic variation in the genus *Lolium*

Species	P	A	H_T	H_S	Marker	References
L. canariense	35%	1.40		0.104	enzymes	Charmet and Balfourier 1994
L. loliaceum	14%	1.14		0.050	enzymes	Charmet and Balfourier 1994
L. persicum	3%	1.03		0.015	enzymes	Charmet and Balfourier 1994
L. remotum	7%	1.07		0.014	enzymes	Charmet and Balfourier 1994
	-	1.20		0.006	enzymes	Loos 1993b
L. temulentum	-	-	-	0.087	enzymes	Bennett et al. 2002
	0	1.00		0.000	enzymes	Charmet and Balfourier 1994
L. multiflorum	-	-	-	0.379	enzymes	Bennett et al. 2002
	91%	2.83		0.392	enzymes	Charmet and Balfourier 1994
	-	3.23		0.384	enzymes	Loos 1993b
	89%	-	0.710	0.700	RAPD	Vieira et al. 2004
L. perenne	-	-	-	0.354	enzymes	Bennett et al. 2002
	76%	2.13		0.273	enzymes	Charmet and Balfourier 1994
	-	2.15	-	0.277	enzymes	Loos 1993b
	-	2.72	0.362	0.322	enzymes	Balfourier et al. 1998
	-	-	0.821	0.492	cpDNA	Balfourier et al. 2000
	85%	2.60	0.353	0.331	enzymes	Fernando et al. 1997
L. rigidum	-	-	-	0.375	enzymes	Bennett et al. 2002
	86%	2.77		0.350	enzymes	Charmet and Balfourier 1994
	-	3.28	-	0.346	enzymes	Loos 1993b
	-	3.12	0.488	0.405	enzymes	Balfourier et al. 1998
	-	-	0.463	0.274	cpDNA	Balfourier et al. 2000

power of DNA markers applied include a total of 775 bands from which 621 are polymorphic that is exactly 80%. The present results are clear. The extent of DNA variation as estimated from the percentage of polymorphic loci, mean number of alleles per a locus and total gene diversity is comparable with that revealed by enzymes thus, confirming the utility of both approaches in population genetic studies of ryegrasses. The major comparison between DNA data (mainly RAPD) and allozyme data conducted by Nybom (2004) also shows that RAPD-derived gene diversity parameters are rather close to the allozyme derived.

Surveys of the majority of marker analysed have revealed nearly the same level of polymorphism in *L. multiflorum* and *L. perenne* as estimated from the overall proportion of polymorphic loci (75% and 72%, respectively), mean number of alleles (1.75 and 1.71) and effective alleles per a locus (1.41 and 1.47). Also the values of genetic diversity are mostly alike (H_T=0.257 and 0.273). The markers specific to a species are hardly found with the only exception of few bands revealed by SSAP. This is in contrast to results of Bennett et al. (2002) who suggested that *L. perenne* does possess less enzymatic diversity that *L. multiflorum*. Balfourier et al. (1998) speculated that this is a result of reproduction system. In *L. perenne* only a small number of plants may participate in reproduction as it is a perennial species used for intensive grazing in natural and permanent pasture. It should be noticed however, that the difference between estimation of the average gene diversity in the work of Bennett et al. (2002) was very small (0.025) and presumably not significant statistically. It is not plausible neither that the perennially can reduce the genetic diversity at all. As it has been

well documented for *Trifolium* (Bulińska-Radomska 2005), longevity has no significant effect on distribution of variation and a wide range of variation was observed in perennial species. Moreover, analyses of extensive data compilations have demonstrated that long-lived perennial taxa have rather higher diversity than annual taxa (Nybom 2004).

The similar level of genetic variation is somewhat unexpected for species that are thought to be adapted for diverse ecological conditions i.e., *L. multiflorum* for mild, warm climate and *L. perenne* for colder, north climate. Very often even closely situated conspecific populations differ in allele frequencies as a result of adaptation to slightly different conditions (soil, shadow etc). The variation that arises within population becomes transformed into variation among populations and species. In wild emmer wheat (*Triticum dicoccoides*), for example, enzymatic and ribosomal DNA polymorphism displays considerable regional and local differences (Nevo 1983; Flavell et al. 1986). The same is true for enzymes and hordein polymorphism in wild barley *(Hordeum spontaneum)*, which is adaptive and selected by soil and topographic differences over very short distances. About 56% of all variant enzymatic alleles are not widespread but reveal localized and sporadic distribution (Nevo et al. 1983). Commonly, the farther apart populations are, the more different they are in genetic structure. However, the results from highly variable, out-crossing species can greatly differ. Populations of distant geographic origin can show a very little divergence if any. This is known for *Pinus sylvestris*, whose populations are very similar on enzymatic level (Goncharenko et al. 1994) as well as on DNA level (Polok et al. 2005a). Based on the present results, two out-crossing *Lolium* species, *L. multiflorum* and *L. perenne* clearly fall within this group of species. Southern Italian ryegrass ecotypes are grouped together with Polish ecotypes of perennial ryegrass based on enzymatic allele frequencies. On the other hand such results understandably attribute to high gene flow resulting from rather sympatric distribution of ryegrasses over the huge temperate areas of Europe, Asia, America and Australia.

While the majority of marker systems used display the same level of polymorphism in *L. multiflorum* and *L. perenne*, there are some exceptions. Unexpectedly low level of cpDNA polymorphism in *L. multiflorum* (40%) has strong roots in extremely low polymorphism of cultivars of this species. Another important differences between Italian and perennial ryegrass i.e., higher transposon diversity in the former may be attributed to the role of transposons in species differentiation from one side and, in the case of *Lolcopia1* to its origin from *L. multiflorum*. Quite different can be the basis of a higher number of ISJ alleles in *L. perenne*. One possible explanation is that it may be a function of the adaptation to more northern environments where the ability to grow during dark periods can decide about reproductive success. North ecotypes of perennial ryegrass are able to produce normal short stems (not etiolated) and opened expanded shoots long in the autumn and even during the whole winter if it is relatively mild. Several evidences from fine-scale mutagenic studies have proved that these features are very often caused by point mutations at intron splice junctions as for example in the light-independent photomorphogenesis mutant *lip1* in pea (Sullivan and Gray 2000). Light-independent forms occur neither in more southern *L. perenne* populations nor *L. multiflorum*. In other words, the presence of light-dependent and light-independent forms in *L. perenne* correlates with mutations in intron splice junctions and this transforms into higher number of ISJ alleles in *L. perenne* because this type of marker reveals polymorphism at intron splice junctions.

Hamrick and Godt have suggested (1989) that the majority of variation in allogamous species is distributed within populations. This appears as relatively high average gene diversity (H_S) and mean G_{ST} value of 0.099 that can be roughly explained that only 9.9% of the total variation is distributed between populations. A subset of enzymatic and DNA data has confirmed high within-population diversity of *L. multiflorum* (Oliveira et al. 1997), *L. perenne* (Fernando et al. 1997; Balfourier et al. 1998) and *L. rigidum* (Balfourier et al. 1998). The among-population diversity has been low and ranged from 8% to 14%. By contrary, all marker types used in the present work, show quite different pattern of variation. The levels of genetic diversities among populations are two-three times higher (50-60%) than those previously reported. It can not be excluded that nonrandom distribution of dominant DNA markers through the genome could distort the results considerable. In general, at least four times more dominant markers are needed to obtain the same efficiency as with codominant ones. With 15 enzymatic loci and the minimum number of 55 dominant ISJ loci this condition has been fulfilled pretty well. It is striking however, that similar results have been observed for codominant enzymatic loci thus, indicating another reason for the discrepancy between the present and literature data. The population genetic parameters are associated neither with the number of populations nor number of plants per population as long as the population size is above approximately ten plants (Nybom 2004). Most of authors cited above used a similar sampling scale. Therefore, either evolutionary or historical processes have shaped the present differentiation of Italian and perennial ryegrass populations. Pros is the high variation between *L. perenne* accessions revealed by RAPDs (Bolaric et al. 2005b), AFLPs (Guthridge et al. 2001) and cpDNA (Balfourier et al. 2000).

The relatively high G_{ST} values reflect spatial genetic structure and suggest restricted levels of gene flow. In many plant species, gene flow via pollen is sufficiently limited to less than a few hundred individuals occupying areas less than 50 m^2. In the grasses *L. perenne* and *F. pratensis*, pollen dispersal decreases rapidly within 75 m from the donor field (Giddings 2000; Rognli et al. 2000). Although any immigrant pollen can be found a kilometer away from the source it is not likely to have an effect on genetic variation. Like *L. multiflorum* and *L. perenne* in the present studies, many wind-pollinated species have a relatively strong population structure suggesting that wind may not be a particularly effective agent for pollen dispersal (Avise 2004). However, given the lack of differences in genetic structure of *L. multiflorum* and *L. perenne*, the hypothesis of limited gene flow does not seem to be the most convincing. Furthermore when among-population diversity is calculated separately on a cultivar and ecotype basis, the G_{ST} value drops down and it fits better with values typical of allogamous species.

Another explanation involves the role of glacial and interglacial cycles. The last glacial period reached a peak between 25 000 and 18 000 years ago. During this period, land temperatures dropped as much as 20°C. These climatic changes led to changes in the distribution of many plant species. Afterwards, in the current interglacial period, species migrated northwards (Roberts 1998). These historical associations among populations, rather than patterns of ongoing gene flow, may play a predominant role in shaping patterns of genetic structure. Molecular analysis of *Abies* species from southern Mexico and Guatemala indicates that these populations may have passed through a number of genetic bottlenecks that led to a loss of genetic diversity and interpopulational differentiation due to genetic drift (Agu-

irre-Planter et al. 2000). This idea has been also illustrated by a study of population differentiation in the Mediterranean *Senecio gallicus* that probably persisted in Pleistocene coastal refugia during glaciation periods. A significant decline in haplotype diversity has resulted from re-colonization from particular refugia and founder events (Thompson 1999). Such a phenomenon has been also observed in *Sorbus aucuparia* with isozyme and cpDNA markers (Raspe et al. 2000) and could also apply to *Lolium*. A comparative summary of allozymes and cpDNA analyses in *L. perenne* and *L. rigidum* leads to two possible scenarios (Balfourier et al. 1998; 2000). Under the first scenario Europe was colonised from the Middle East before the last glaciation, then after *Lolium* extinction during the glaciation, Europe was re-colonised from several postglacial refugia. Frequent extinctions and re-colonisations might increase genetic differentiation among newly founded populations. The second scenario matches the historical processes such as the emergence of agriculture. Ryegrass populations came into Europe with the first farmers as weeds of cereal crops. Selection pressure effects after migration might then explain the present differentiation of populations. The organelle DNA data presented here in conjunction with information from nuclear markers indicate that both scenarios contributed to the distribution of *Lolium*. The presence of private cpDNA and mtDNA haplotypes in the *L. perenne* ecotype from the Tatras suggests that this population has been genetically isolated for a considerable period of time. It was collected in the Chochołowska valley. In the lower Pleistocene, about 2.0-0.7 million years ago, the Tatra climate was moderate thus, allowing temperate species to develop over wider areas than at present. Grasses were a prominent component of the Tatra plant communities at that time. From about 700 000 to 10 000 years ago the Tatra Mts. underwent at least three glaciations. However, in contrast with the Alps, relatively small areas were covered by glacier. In particular, the lower parts of west valleys i.e., Bystrego, Malej Laki, Mietusia, Kościeliska, and Chochołowska were not glaciated (Klimaszewski 1996; Obidowicz 1996) allowing grasslands persist. Thus, perennial ryegrass populations in the Tatras could have been fragmented, becoming smaller and more isolated but they might survive the glaciation periods. Similarly, there is increasing evidence for full-glacial tree growth in central and eastern Europe (Willis and Niklas 2004). Furthermore, due to difficult living conditions, and following a very early legal protection, the Tatra nature has not been disturbed for centuries. The traditional agriculture has prevented the extinction of old genotypes by crossing with modern cultivars, alien to the Tatra flora. It can be argued therefore, that the Tatra ecotype represents rather a relict from the period before glaciations than a colonizer from southern refugia. A somewhat separate position in *Tpo1* and *Lolcopia2* dendrograms as well as the presence of a unique peroxidase allele *(Per1-45)* is also some support for this view. The second point of note concerns Scandinavian ecotypes of *L. perenne* that share mtDNA haplotypes with Italian ecotypes of *L. multiflorum*. Such a distribution of haplotypes could be explained by human-mediated migration of populations into Scandinavia from the south Europe. These studies thus, provide a clear illustration of how populations in different regions may follow different evolutionary trajectories.

4.4.2. Genetic variation of cultivars and ecotypes

There are further reasons why the strong differentiation of populations has been observed. All populations show an excess of homozygotes as estimated from FIT. Significant

deviations from expected heterozygotes frequencies, as observed in the present study, have often been related to inbreeding. This should not be surprising given the fact that ryegrasses are undergoing the intense breeding. *Acc*ording to the Convention of the International Union for the Protection of New Varieties of Plants (UPOV 1990) each new cultivar must be distinct from every previously registered, stable through cycles of multiplication and uniform in its characteristics. During the selection process breeders have to consider these requirements. In particular, the potential parents for a cultivar are selected for uniformity from a limited number of clones. Some cultivars have been developed from several plants of a single ecotype collected from a very restricted area (Bolaric et al. 2005b). One striking result to emerge from the organelle DNA data presented here is the small variation in cpDNA (P, HT and HS), especially in cultivars of*L. multiflorum* and *L. perenne* that would imply for one or very few material origins. The same is true for other grass species such as *Buchloe dactyloides* (Gulsen et al. 2005). Given the slightly lower polymorphism of *L. multiflorum* and *L. perenne* cultivars revealed by ISJs and *Lolcopia2*, their narrow genetic background seems to be plausible.

On the other hand, one could argue that these differences are small and the majority of markers used reveal the same level of genetic diversity in cultivars and ecotypes. Thus, plant breeders' activities have maintained the level of molecular variation present in ecotypes. This is important conclusion with regard to more and more intensive land use. Cultivars have been widely spread by the seed trade. And as pointed out by Bolaric et al. (2005b), during the past 40 years a large area of old permanent grassland has been re-sown with new cultivars. Heavily grazed pastures are re-established every 3-5 years and consequently, a large number of ecotypes have been ousted. Moreover future cultivars are predicted to be based on much narrower genetic basis because they will be the most suitable for molecular breeding allowing near completediscrimination (Guthridge et al. 2001). Hence the important issue is to capture natural genetic diversity to sustain future breeding objectives. It is clear both from the present data and literature review (Bolaric et al. 2005b) that *L. perenne* cultivar-breeding programmes have undertaken this challenge with success. Molecular diversity of *L. perenne* cultivars overlaps this of ecotypes. The use of genetic markers, in particular maternally inherited organelle DNA and its comparison with nuclear genes, has allowed for the examination of breeding history of cultivars. For example Portstewart (*L. perenne*) shares the cpDNA haplotype with Scandinavian ecotype, NGB10809 thus, suggesting their common origin. There is additional support for this thesis from enzymatic data. Evidences both from cpDNA and mtDNA indicate that the other two *L. perenne* cultivars, Lisuna and Magella may be derived from the second Scandinavian ecotype NGB4264. In fact, these results are consistent with the UPGMA grouping based on enzymes and ISJs. Thus, perennial ryegrass cultivars remain the most similar to ecotypes of this species although the introgression from *L. multiflorum* can not be excluded as concluded from *Tpo1*. This DNA transposon reveals significantly higher diversity in *L. multiflorum*. *L. perenne* cultivars have the values intermediate between *L. multiflorum* and *L. perenne* ecotypes.

The data presented demonstrate clearly that breeding strategies are slightly different in *L. multiflorum*. Despite similar level of genetic diversity in cultivars and ecotypes, the former occupy a separate position in UPGMA dendrograms and multidimensional scaling scatterplots whereas the latter tend to group together with *L. perenne*. The pattern of ge-

netic diversity of *L. multiflorum* cultivars appears to be related to morphological variation. All *L. multiflorum* cultivars analysed here by means of molecular markers are also clearly distinct from ecotypes by extremely high values for generative traits (Chapter 3). Examination of cp and mtDNA haplotypes shows that Bartissimo appears to be derived from Variamo (the same cpDNA and mtDNA), Bartolini from Guliamo whereas a distinct mtDNA haplotype in Atalja indicates different origin. However, each cultivar has specific haplotypes not present in the other *Lolium* in addition to significantly higher frequency of several RAPD bands (e.g., OPA02-4). Based on their morphological performance the introgressions from the other closely related species e.g., from the genus *Festuca* is proposed (Chapter 3). The molecular data seems to support this hypothesis. However, it must be emphasized that these interpretations are preliminary and further organelle DNA data based on a wider primer and species (e.g., *Festuca*) choice, will be needed to build up a definitive picture.

4.4.3. Phylogenetic relationships between *L. multiflorum* and *L. perenne*

The most striking results to emerge from the present work are high genetic similarities between *L. multiflorum* and *L. perenne* more typically associated with conspecific populations. Huge genetic identities are apparent in nuclear, chloroplast and mitochondrial genomes (I ranged from 0.986 to 0.887). This is not unexpected because the high level of similarity has been observed by the other authors as well (Bulińska-Radomska and Lester 1985; Loos 1993b; Bennett et al. 2002). However, most of these data were obtained by enzyme electrophoresis and the authors believed that due to low resolution it would not be possible to make more precise picture until advanced DNA methods would be available for forage grasses (Charmet and Balfourier 1994). Although several DNA-based comparisons have been done for Italian and perennial ryegrass (Stammers et al. 1995; Zielinski et al. 1997; Balfourier et al. 2000) none of these research are comparable with the present work both with reference to the diversity of markers applied and plant material including cultivars and wild ecotypes. Another important point to notice is the approach in which the same plant individuals have been examined by all marker categories. This enables to compare the results obtained with different methods on the same plant material. And the results are clear - the enzymatic data have not been cheating - high similarity is observed at the DNA level irrespective of the marker type used. Moreover, the gene identities obtained by different methods are very alike. Even values based on insertional polymorphism that is thought to be highly differentiating, are well ahead those for different species confirming the extensive gene flow between both species.

Considerations about the meaning of high similarity between *L. multiflorum* and *L. perenne* inevitable give rise to the question how much genetic exchange disqualifies populations from status as separate biological species. Biological species concept (BSC) perceives species as populations between which the gene exchange is limited or prevented by reproductive isolating mechanisms (Avise 2004). One difficulty of BSC involves the primacy of reproductive barrier in demarcating species and thus, no arbitrary magnitude of molecular genetic divergence can provide infallible metric to establish specific status. How, then, can the molecular data inform about speciation of ryegrasses?

One possibility is the estimation of genetic identity. The concept of genetic identity/distance is fundamental to evolutionary studies. A genetic identity (I) between two sequences,

individuals, populations or taxa is a quantitative estimate of how similar they are genetically. The converse of genetic similarity is genetic distance (D) that measures the extent of gene differences. When genetic distance is low, genetic similarity is high, and vice versa. The expectation is that genetic identity gradually decreases with increasing time of divergence because of different mutations accumulating in the two populations/species (Nei 1987).

In the past thirty years a wealth of data has accumulated concerning the genetic identity (distance) between taxa of various ranks. From these studies, some general features have emerged. The genetic identity between local races varies from 1.00 to 0.90 and is generally much larger than that for interspecific comparisons. The genetic identity between well-defined biological species is generally low and varies from 0.20 to 0.35 (Zielinski and Polok 2005). The process of divergence is continuous, proceeding both before and after speciation. Thus, between the I value typical of conspecific populations and well-defined biological species is a huge gap, informing about the stage of divergence. The classical example of the gradual decrease of I value has arisen from *Drosophila willistoni* complex enzymatic studies. Thus, genetic identity of 0.970 is typical of geographic populations that are fully compatible reproducible. Semispecies or species at the early stage of divergence that overlap in geographic distribution and show both postzygotic and prezygotic reproductive barrier have I equal to 0.873, whereas subspecies, that are allopatric and exhibit incipient postzygotic barrier, - 0.795. Fully isolated but nearly identical morphologically sibling and nonsibling species that are phenotypicallydistinct differ more appreciably with I value 0.517 and 0.352, respectively (Futuyma 1987). Subsequent analyses of more pairs of closely related species demonstrated that even partial reproductive isolation is often associated with large genetic distances (Avise 2004).

As judge by the value of genetic identity, *L. multiflorum* and *L. perenne* should be classified as a single species, eventually as semispecies or species at the very early stage of divergence. Even the subspecies status currently recommended by the Integrated Taxonomic Information System of the USA (2007) seems to high. Although such suggestions are not new in their original formulation (Naylor 1960; Bulińska-Radomska and Lester 1985; Zielinski et al. 1997) the present data provide the strongest support for this hypothesis. There are dozens of additional examples of species re-classifications following molecular analysis. *Aconitum noveboracense* and *A. columbianum* are herbaceous perennials that occur in the USA. The former is a rare species while the latter is a much more common species in particular in mountainous areas. Likewise ryegrasses, these two species can not be reliable differentiated based on morphological characters. Molecular analyses that revealed significant similarities in allozyme and RAPDs ($I \geq 0.90$) have championed their treatment as a single species (Cole and Kuchenreuther 2001). Remarkable examples of high molecular similarities despite morphological differences are provided by *Pinus cembra* and *P. pumila*, of which the latter is supposed to be a dwarf mutant of the former (Polok et al., unpublished data). A peat-bog pine, *Pinus uliginosa* has been thought to be a hybrid between *P. sylvestris* and *P. mugo*, but a battery of DNA markers have proved instead that it is closely related (I=0.90) with *P. mugo* with which it shares a gene pool (Polok et al. 2007).

Genetic similarity provides, therefore, a valuable service to ryegrass re-classification. However, several cautionary points should be made about such mechanistic appraisal. Speciation is highly variable process, differing greatly in mean tempo and mode in different kinds

of organisms. Many authors have concluded that *L. multiflorum* and *L. perenne* are indeed distinct in spite of high allozyme similarity because there are few statistically significant differences in frequency of enzymatic alleles. They are also distinguished by taxonomists based on morphology (Loos 1993a; b; Bennett et al. 2002). One intriguing possibility is that insufficient time has elapsed for accumulation of greater *de novo* mutational differences. Indeed, the time-depended aspect of allozyme divergence permitted reassessments of speciation dates. For example, *L. multiflorum* and *L. perenne* prove to exhibit an allozyme similarity of 0.98 that is equal to genetic distance of 0.02. If we assume that the average codon substitutions per a locus per a year that is detectable by enzyme electrophoresis is 10^{-5}, the probable time of their divergence is only 3000 years. If this value is correct, then it fits historical processes such as the emergence of primitive agriculture in the Middle East and its expansion towards Europe. The record for magnitude of genetic similarity among taxonomic species that has been considered conspecific involves the *Agave deserti* complex, representing a group of species and subspecies with near allopatric distribution and clear differences in morphology. The average Nei's genetic similarity based on RAPDs between species is 0.96 and no correlation with taxonomic division is observed (Navarro-Quezada et al. 2003). Other noteworthy studies using allozymes have demonstrated little genetic differentiation between two subspecies of herbaceous plant *Delphinium variegatum* (Dodd and Helenurm 2002). However, the taxonomic revision has been proposed in most such evidences. Furthermore, it is important to bear in mind that some taxonomists do not use the biological species concept to define their taxonomic species, but rely on morphological differences, without explicitly considering whether or not morphological differences provide evidence for reproductive isolation. From the data presented here, it can be the case of *L. multiflorum* and *L. perenne* as well. Despite a huge amount of data demonstrating that they posses the same gene pool, that there is no a reproductive barrier, for years taxonomists have been used to classify them as distinct species.

Among other explanations, the so called "sudden speciation" that entails little or no change in genetic composition at the allelic level should be taken into account. Is such a scenario plausible for *L. multiflorum* and *L. perenne*? Recent theories have stressed several possibilities by which species can arise rapidly with minimal molecular genetic divergence overall. Several known pathways include polyploidization, chromosomal rearrangements, and changes in mating system.

First, polyploidization is the best known mechanism of sudden speciation and usually is associated with hybridization between species that differ in chromosomal constitution. The hybrid sterility is removed then by the doubling of chromosomes and a new polyploid hybrid is produced. Although it is a very frequent mechanism in plants (Zielinski 1987; Ramana and Jacobsen 2003), it is not a case of *L. multiflorum* and *L. perenne* because they both normally are diploids with the same chromosome number.

Second, closely related taxa may differ in a variety of structural chromosomal features including micro-deletions, translocations, inversions that may cause improper chromosome pairing, disjunctions during meiosis resulting in slightly lower hybrid fertility. Although it has previously been shown that hybridization between *L. multiflorum* and *L. perenne* results in some loss of fitness (Naylor 1960), it has not been confirmed and a lot of hybrid cultivars are characterized by high vigour and fertility. Structural rearrangements

(inversions) in specific chromosomal regions have the effect of reducing recombination when in heterozygous conditions. This reduction can act as a partial barrier to gene exchange in genomic regions that differ karyotypically. For instance, several large-scale chromosomal rearrangements are observed between ryegrass and Triticeae species (Sim et al. 2005). *Arabidopsis thaliana* and *A. lyrata* were separated roughly 5-6 MYA and the former underwent a dramatic genome reconstruction with approximately 1.3 chromosomal rearrangements per million years (Koch and Kiefer 2005). Among others, transposons are one of the most important factors inducing chromosomal rearrangements (Zhang and Peterson 2004). However, prior to detailed mapping studies this possibility can neither be excluded nor confirmed.

Finally, many plant species exhibit partial self-incompatibility (SI) that involves molecular recognition events between pollen and stigma determining whether pollination event will produce seeds or not. It is a major pre-zygotic pollination barrier which, although primarily considered as an intraspecific barrier, is also important in the prevention of hybridization between closely related species. Self-incompatibility system in ryegrasses has attracted interest mostly because the attempts to produce self-fertile lines as parents to F_1 hybrid cultivars. Likewise many other grasses, *L. multiflorum* and *L. perenne* exhibit two locus self-incompatibility system (S and Z) in which the two loci are complementary in action (Thorogood et al. 2002). However, it has never been considered as a possible barrier for interspecific hybridization. Such seems to be the case present in maize and its wild relatives. Annual Mexican teosinte, *Zea mays* subspecies *parviglumis*, and subspecies *mexicana* grow wild in disturbed habitats including cultivated maize fields. As a cultigen, maize has been favoured by human activity whereas, as a weed, teosinte has been subjected to natural selection. Yet, their karyotypes are similar, pollen fertility of F_1 hybrids is high, and the level of recombination is similar to that in maize. However hybrids are rarely produced in nature and poorly adapted. This is attributed to the *Tcb1-s* locus that is polymorphic in wild teosinte populations but only recessive homozygotes (*tcb1 tcb1*) are observed in cultivated maize. Maize can only be fertilized by teosinte pollen carrying *tcb1*. The dominant allele is highly abundant in wild populations so the possibility of hybridization with maize is very low (Kermicle 2006). Alike maize, various counterselective forces have acted on *L. multiflorum* and *L. perenne* during their evolution. Their karyotypes are similar as well and pollen fertility of F_1 hybrids is high. On the other hand it has been suggested that interspecific hybrids are rarely produced in nature due to reproductive agronomical isolation (Arcioni and Mariotti 1983). Although there may be plenty explanations of this, one might imagine the role of self-incompatibility loci in the emergence of the crossing barrier and in facilitating adaptation of these taxa to the different habitats. Whether this explanation is plausible or not, it is possible to learn in more detailed genetic studies. The significant distortions observed for markers linked to self-incompatibility loci in interspecific crosses will be a sign of their role in the establishment of crossing barriers. Indeed, the S and Z loci have been mapped to linkage group one (LG1) and two (LG2), and markers closely linked with them do not show Mendelian segregation in *L. perenne* (Thorogood et al. 2002; 2005).

The present findings demonstrate the discrepancy between low divergence of *L. multiflorum* and *L. perenne* and their taxonomic status. It can be argued after Nei (1987) that species with I values about 0.85 should be considered doubtful if there is no other evidence of their specific status. The common gene pools, lack of species specific markers, the good

agreement between molecular and morphological diversity (Chapter 3) and high inter-fertility of both species as reported by breeders champion strongly for lowering their taxonomic rank. It should be noted however, that prior to more detailed mapping studies some mechanisms of "sudden speciation" can not be absolutely excluded. Especially, that the lower transposon-based similarity in comparison with the other markers might be the first signs of speciation.

4.4.4. Role of transposons in differentiation of *L. multiflorum* and *L. perenne*

Mobile genetic elements also called transposons or transposable elements (TE) are prevalent in the genomes of all plants contributing up to 90% of the genome (Kazazian 2004). Most of the moderately repeated sequences are mobile genetic elements. Transposons are classified into two groups according to their transposition mechanism and mode of propagation. Class I elements (retrotransposons) move via an RNA intermediate that is reverse transcribed prior to integration into the genome ("copy-paste"). Once inserted, they can not excise. This mode of reproduction contributes significantly to the genome expansion. Retro-elements are usually flanked by long terminal repeats (LTRs). In plants, the members of two groups are predominately observed; the most studied *Ty1-copia*, to which *Lolcopia1* and *Lolcopia2* belong, and *Ty3-gypsy*. Both groups were named after the best studied elements in *Saccharomyces cerevisiae* and *Drosophila melanogaster*. Plant *Ty1-copia* retrotransposons show a considerable degree of sequence heterogeneity compared to fungal and animal elements. Class II elements, often termed DNA transposons, are excised from the genome and integrated elsewhere ("cut-paste"). They typically possess terminal inverted repeats (TIRs) on the basis of which they have been subdivided into several super-families. One of them, called the CACTA family, received its name because it is flanked by inverted repeats that terminate in a conserved CACTA motif. The *Tpo1* transposon analysed in the present studies belongs to this super-family (Flavell et al. 1992; Turcotte et al. 2001; Vershinin et al. 2003; Wicker et al. 2003). Transposons generate genetic diversity by altering the size and organization of the host genomes and by inducing insertional mutations. The evidence from *H. spontaneum* has indicated that both active amplification and losses of transposons are likely to generate genome diversity (Kumar and Hirochika 2001). A multiplex PCR approach, SSAP has been developed to detect insertional polymorphism of transposons in plants. In SSAP the products are derived from a DNA fragment between retrotransposon terminal sequences and a restriction site in the sequence flanking the transposable element thus, the method reveals the sites of transposon insertion.

Retrotransposons make up a large proportion of the genome of higher plants and they contribute to more than 50% of the nuclear DNA being present even in millions copies. They are commonly found in intergenic regions what reduces their detrimental effect (Bennetzen 2005). This is not surprising therefore, that *Lolcopia1* and *Lolcopia2* retrotransposons are highly abundant in *L. multiflorum* and *L. perenne*. One of the most convincing cases of *Lolcopia* abundance in ryegrasses involves the comparison with the other species. The only three primer combinations have revealed from 160 to 251 insertion sites and it is four times more than it is observed in tomato (46) and pepper (40) with the same number of primer combinations (Tam et al. 2005). The present data indicate as well that primers designed on the basis of 3'region of LTR sequences derived from other Poaceae species can be used in

related genera of the same plant family. The *Lolcopia1* is amplified in both Italian and perennial ryegrass although the primer was designed on the basis of *L. multiflorum* and *O. sativa* LTRs. Similarly, *Lolcopia2* based on *S. officinarum* and *T. aestivum* LTRs is also present in ryegrasses.

In opposite to retrotransposons, DNA transposons predominate in or around genes, increasing the potential phenotypic impact of individual movements. That is why they are usually less abundant in the genome with the copy number from ten to fewer to hundreds (Langdon et al. 2003). Unlike the typical case for DNA transposons, *Tpo1* is present at a sufficiently high density in *L. multiflorum* and *L. perenne* genome. The copy number is similar to retrotransposons. This observation is supported however by congruent results obtained from *L. perenne* (Langdon et al. 2003) and Triticeae (Wicker et al. 2003). The *Tpo1* family is a lineage of CACTA superfamily whose members are unusual in consistently having a high copy number in species with large genomes. The reasons behind a mechanism accounting for the host's ability to tolerate large numbers of *Tpo1* elements have not been understood so far. One speculative explanation that can be also adopted for the present results includes the insertion in an active retrotransposon. In that way the *Tpo1* ancestor could have become dispersed to large numbers of intergenic regions at a rate far higher than could be achieved by conventional transposition. The presence of an RN-aseH-like motif that is typical of retrotrasposon accounts for the above hypothesis (Langdon et al. 2003).

Being highly abundant in plant genomes, transposons are one of the most important factors affecting the population structure of a species. The transposition process produces hundreds of thousands of new insertions in the genome, comprising the driving force of polymorphism acquisition. These genetic properties have been exploited to study genetic diversity in *L. multiflorum* and *L. perenne*. Unsurprisingly, the insertion sites of all transposons analysed are highly polymorphic within each species. This is rather a common feature of transposon-based population genetic parameters as can be deduced from the noteworthy studies in pea (Vershinin et al. 2003), soybean (Chesnay et. al. 2007), barley (Leigh et al. 2003) and many others (Gribbon et al. 1999). Thus, the crucial question is not what the level of insertional polymorphism is but what its magnitude in comparison with the other marker categories is. Unfortunately, such analyses have been hardly done and the present work is the one of just a few. One notable study demonstrating much higher transposon-based polymorphism than AFLP involves tomato and pepper (Tam et al. 2005). In tomato SSAP has shown threefold more diversity than AFLP (P=57 and 15%, H=0.175 and 0.046, respectively). Even more dramatic, tenfold difference has been observed in pepper (P=76 and 8%, H=0.229 and 0.026). Another example has shown that in *Pisum* the SSAP approach based on *PDR1* retrotransposon is more informative and generates more polymorphism than AFLP (Kumar and Hirochika 2001). The difference is less pronounced in sweet potato, in which SSAP reveals 18% more polymorphic bands than AFLP and 10% more than RAPD (Berenyi et al. 2002). These data are in apparent contradiction to the examples from *L. multiflorum* and *L. perenne*, of which the magnitude of transposon-based genetic variation (P=60-83%, A=1.6-1.8) and this reveals by the other nuclear DNA markers and isozymes (P=75%-96%, A=1.7-1.9) are very alike. In a sense, the present studies merely affirm that the level of polymorphism appears not to be attributable to a marker type. The transposon-based parameters are not biased and can give a good picture of species diversity. Additional advantage of

SSAP in comparison with the other markers is that only several gels are needed to gather huge amount of data.

Transposons are one of the most variable of genomic components, being the main players in the evolution of plant genomes. During evolution the number of copies of transposable elements has been changing resulting in the loss or gain of transposon insertion sites and finally resulting in changes to the SSAP profiles. SSAP has proved to be the most efficient marker system for studying phylogenetic relationships between *L. multiflorum* and *L. perenne* as estimated from the lowest genetic similarities in comparison with the other markers and the best separation of both species in the dendrograms and multidimensional scatterplots. SSAP technique has also been useful in estimating phylogenetic relationships in the other Poaceae. In the *Zea* genus, the Grande retrotransposon-based phylogeny has reconstructed the established taxonomy at the species and subspecies level (Garcia-Martinez and Jose-Izquierdo 2003). The patterns of site variation, termed the transposon signature, have been used to examine the relationships within the genera *Zea* and *Tripsacum* (Kumar and Hirochika 2001). Utility of SSAP in studying a species diversification has been reported for wheat and barley (Gribbon et al. 1999; Queen et al. 2004). Nevertheless, transposons have been the most successful in estimating phylogenetic relationships in *Pisum*. The retrotransposon-based markers give a clear separation of the main lineages and distinguish the truly wild form of *P. elatius* from the antecedents of *P. sativum* (Vershinin et al. 2003).

One of the most prominent results clearly arising from these studies is that *L. multiflorum* and *L. perenne* have very similar transposon populations. The SSAP profiles produced 640 bands with only 12 being unique for these species. When the sites whose frequency is statistically different between species are taken into account as well, the band sharing is still exceptionally high (95%). Although it is possible that a few universally shared bands may have arisen independently in each species through size homoplasy, this is unlikely to have happened for more than 600 bands. Such a high band sharing is rather unexpected for different taxa. In large genome species the transposable elements are usually highly diverged, even between close relatives (Bennetzen 2005). High levels of insertional polymorphism within *L. multiflorum* and *L. perenne* for all transposons studied suggest strongly that they have been transposing very recently on an evolutionary time scale. Retrotransposon once inserted does not change its position during the evolution of the genome but every insertion elevates the polymorphism. If there was an event of transposition in a lineage leading to one of these ryegrasses, all populations should have this insertion whereas none insertion should be present in another species. An illustration is the SIRE-1 retrotransposon that has been evolving independently in the annual species, *Glycine max* and *G. soja* producing many new retroelement subgroups in each species (Chesnay et al. 2007). In the case of *L. multiflorum* and *L. perenne* only 2% of bands are unique for a species. On the other hand, if insertion took place during population divergence, it should be present in some, in the others it should be lacking. Although this pattern of transposon divergence during evolution is far from being universal, it is prevalent enough to suggest that transposon divergence in Italian and perennial ryegrasses follow the pattern typical of conspecific populations.

Overall, with regard to shared bands, they may represent transposon insertions that occurred in the common ancestor and have been preserved in each descendant lineage. The *BARE-1* retrotransposon, for instance, is broadly distributed among the Triticeae spe-

cies and shared bands occur in *T. aestivum*, *S.* cereale and *A. sativa* (Gribbon et al. 1999). Common bands are observed not only for Triticeae but also for timothy, oat and cordgrass of the subfamily Chloridoideae. Presumably, the integration events took place in the last common ancestor of Poideae and Chloridoideae, long before the Triticeae diverged (Vicient et al. 2001). However, only seven shared bands have been observed in the former example, and probably the similarly low number has been present in the latter. An important drawback to both studies is the lack of data about frequency of shared bands and it can be only deduced from gel images. Assuming that an average bands' number on the SSAP gel is about 40-50, then the frequency of common bands in cited examples is about 14-17%. This magnitude is more or less in agreement with the only published frequency data for the *Glycine species* (Chesnay et al. 2007). The SSAP profile of *SIRE-1* has produced 21% of shared bands in annual accessions and 27% in perennial accessions i.e., almost fourfold less than in the present studies. Considering the above values, it is hardly imaginable that almost all insertions produced by three different transposons in *L. multiflorum* and *L. perenne* are derived from the common ancestor. A follow-up question is whether the time period of evolutionary history of ryegrasses roughly estimated between one million and 10 000 years is sufficient for accumulation of a great number of new insertions. For instance, the transposon population is completely different in sorghum and maize but they had shared last common ancestors less than 15 million years ago (Bennetzen 2005). The *Pisum* retrotransposon *PDR1* has been transposing within roughly the last five MYA, with a peak at 1-2.5 MYA but younger insertions were also found in small subset of accessions (Jing et al. 2005).

A lesson learnt from the genus *Pisum* is that extremely high diversity both within a species and between species have been produced during only "minutes" on the evolutionary timescale providing the transposons could have been acting for a sufficient period of time and their activity has been combined with the other genetic processes such as introgression. Cultivated pea (*P. sativum*) is an Old World legume whose evolutionary history is about 10 000 years although the whole genus is much older. Likewise ryegrasses, existing taxonomic classifications of the genus are not congruent and they distinguish from two to six species including *P. abyssinicum*, *P. elatius*, *P. fulvum*, *P. humile*, and *P. sativum.* Molecular appraisals based on the two major groups of retrotransposons, *PDR1* (*Ty1-copia*) and *Cyclops* (*Ty3-gypsy*), together with *Pis1*, a member of the CACTA super-family, have shown clear separation of the *P. fulvum* lineage (Vershinin et al. 2003). This species has 77 insertion sites not found in any other species (i.e., six times more than between *L. multiflorum* and *L. perenne*) and occupies the separate position on the trees. Another interesting finding is the absence of common markers shared by *P. abyssinicum* and *P. sativum* showing that they were brought into cultivation independently. Thus, transposons have demonstrated that the widely accepted view of *P. abyssinicum* as an ecotype of *P. sativum* does not mirror the evolutionary history of these otherwise different species. On the other hand, the shared insertional polymorphism between the other three *Pisum* species, *P. elatius*, *P. humile* and *P. sativum* and virtually the lack of species specific markers indicate extensive and intermixing gene flow among them. This picture seems to be very similar to that in *L. multiflorum* and *L. perenne*. According to the idea expressed by Vershinin et al. (2003) for pea, it can be assumed that recombination, introgression, and segregation have been responsible for the extremely high fraction of shared bands in ryegrasses preserving

from fixation of insertion sites. If we assume that the degree of divergence of transposon populations between a pair of closely related species is proportional to the evolutionary distance between them (Kumar et al. 1997), we should agree that the evolutionary distance between *L. multiflorum* and *L. perenne* is minimal if any. Following the philosophy of Vershinin et al. (2003), who applied the term species complex to *P. elatius*, *P. humile*, and *P. sativum* as they can hardly be treated as separate taxa, obviously the similar conclusion should be drawn for *L. multiflorum* and *L. perenne*. Thus, experimental evidences both for pea and ryegrasses again show that species concepts and taxonomic protocols impinge considerably on perception of "speciation" process.

Despite huge amount of shared bands and the high transposon-based similarity of *L. multiflorum* and *L. perenne*, it is worthy to notice some minor differences in the level of polymorphism and gene diversity possible suggesting the beginning of diversification. In grasses transposons are main players of evolution shaping both genes and the whole genome. The EST database searching suggests that retrotransposons are generally more active in the grasses than in other groups of plants. The monocots show the highest average fraction of retrotransposon-containing ESTs (Expressed Sequence Tags), 1.75%, compared with 1.40% for the conifer species and 0.92% for the dicots. In barley, the *BARE-1* family is transcribed and translated in somatic tissues. Antibodies to the capsid protein GAG of *BARE-1* detect immunoreactive proteins also in the other species of the tribe Triticeae as well as in *A. sativa* and *O. sativa* (Vicient et al. 2001). The grass clade seems to be in a dynamic period of genomic restructuring (Wessler 2001). And there are no doubts that active transposons exist also in *L. multiflorum* and *L. perenne* that can be indirectly implied from the extremely high polymorphism of all transposons in both species. There are no reasons to believe that transposons have not been driving further evolution of ryegrasses shaping their genomes. This tendency is well supported by lower transposon-based genetic similarities between species in comparison with the other DNA markers and statistically significant differences in gene diversity parameters. Noteworthy, the SSAP is the only method enabling to find species specific markers.

Assuming *L. multiflorum* and *L. perenne* have been diversifying, an emerging question is how their future will look like. This indeed provocative question firstly needs a deep look into the history of ryegrasses printed in a most abundant piece of the genome. Each transposon has been telling its own transposition history, however when a variety of different elements is analysed together they can collectively unmask the genetic history of a species. What then is the picture emerging from transposons?

A basic point about *L. multiflorum* and *L. perenne* is not only that they are not separate species at present, but more important, that they have never been distinct species as can be read from the more ancient history of ryegrasses recorded by the *Lolcopia2*. The same number of insertion sites inevitable suggests that this transposon is either inactive at present or its activity is very low. It likely represents the older lineage inherited after the last common ancestor. The high polymorphism revealed by *Lolcopia2* suggests that it was active in a recent past. And finally, the lack of fixed bands for each species is consistent with the possibility that gene flow between *L. multiflorum* and *L. perenne* is the dominant factor of diversity generation. Such idea disagrees with the widely accepted view that *L. multiflorum* and *L. perenne* used to be separate species. Even some authors agree that

their species boundaries are not distinct at present (Loos 1993b; Charmet and Balfourier 1994), they tend to connect this phenomena with changes in the pattern of agriculture. More generally, Bennett et al (2002) hypothesised that due to the sowing beyond their natural species distribution, they come into contact. The hybridisation in these new environments is increasing and leading to unification of both species into a single entity. This opinion postulating a kind of "reverse evolution" although debatable in interpretation, has raised for the first time an important general point: *L. multiflorum* and *L. perenne* are undergoing further dynamic changes.

The DNA transposon, *Tpo1* and the retrotransposon *Lolcopia1* seem to be the most important drivers of these changes. The copy number of *Tpo1* reflects a slow, but significant increase in *L. multiflorum* in comparison with *L. perenne*, especially evident when wild ecotypes are compared. Dramatic evidence for genome increase in the former species also emerged from *Lolcopia1*. About 40% more copies of this retrotransposon have been observed in *L. multiflorum*, suggesting that *Lolcopia1* is active in this species. Although it would be necessary to make comparisons to the other members of the genus and its closest relatives to assess the contribution of these transposons to genome evolution of *Lolium*, it has become clear that*L. multiflorum* represent a more recent lineage.

Hence, the phylogenetic history and future of *L. multiflorum* and *L. perenne* can be demonstrated as follow. Considering high band sharing and the lack of differences in *Lolcopia2* alongside with data from the other markers used in the present studies *L. multiflorum* and *L. perenne* should be regarded as "species complex" at the very early stage of divergence. *L. perenne* might represent the more ancient lineage judging from *Tpo1* and *Lolcopia1*. This opinion is in agreement with old concepts suggesting that ancestral forms of *Lolium* resembled the present *L. perenne* (Thomas 1981; Bulińska-Radomska and Lester 1988). Based on the known *Lolium* history and the data from transposon analysis, it can be postulated that ancestral "species complex", similar to *L. perenne*, colonized Europe before the last glaciation event in the Quaternary (Balfourier et al. 2000). As discussed earlier it survived the last Ice Age in refugia distributed not only in Near East and the three southern peninsulas of Europe (Balkan, Italian and Iberian), which were free of ice sheets, but also in more northern regions. Indeed, there are at least 35 localities from central and eastern Europe proposed to be the full-glacial refugia (Willis and Niklas 2004). The presumably more diffuse distribution of species complex during the cold stage than previously thought entailed that some populations were not sufficiently isolated for diversification to have occurred. This history is recorded by *Lolcopia2* and several other markers that do not diversify *L. multiflorum* and *L. perenne*. Moreover, as cpDNA analyses have shown, the Tatra ecotype might be a relict from those remote times. In recent years, much has been learnt about the role of multiple glacial-interglacial cycles in the Quaternary period in biota evolution. Until recently, it has been believed that the predominant response of species to habitat fragmentation was extinction with speciation taking place in isolated populations and then re-colonisation into north and east after the last glaciation. This scenario has been also proposed for *Lolium perenne* (Balfourier et al. 1998; 2000). Nevertheless, it has become far clear that responses of plant species to the Quaternary Ice Age were complex. Although some species became extinct, others survived the full-glacial periods including many tree species (Willis and Niklas 2004). So did *Lolium*. Having in mind the *Tpo1* and *Lolcopia1* evidences, it is reasonable to specu-

late that the species complex has started to diversify long after the last glaciation. This process appears to be attributable to more recent climatic changes. Strangely, but the climatic conditions in the glacial periods could be more favourable to *Lolium* than they are today. Despite overall temperatures were lower, glacial winters were less severe, while glacial summers were more moderate than present day seasons are in central, north and east Europe. Some regions during the periods of glacial maximum were significantly moister than they would be today (McDonald et al. 2000). Assuming the lack of agriculture in Europe, today the majority of its area would be covered by temperate forests. However, the primitive agriculture started to develop 10 000 years ago following by broad scale deforestation that become significant about 4000 years ago. Since that time the average global temperature has increased of 4.5°C (Ray and Adams 2001). At present, in Mediterranean countries the agricultural environment is severely affected by water shortage in addition to increasing frequency of hot and dry summers. Populations that are predominantly sown in southern Europe appear to adapt to this changing conditions by systematic reorganisation of the genome. Thus, we are witnessing the separation of *L. multiflorum* that stems from the species complex precedent. The changes are discrete, not visible at the level of genes and even not enough to acquire a reproductive barrier. However, they are enough fast to be recognised on the transposon level and to increase the genetic variability as an answer the drought stress. Accumulated data have indicated that transposons respond to various environmental stresses. For example, in *H. spontaneum*, a simultaneous increase in the *BARE-1* copy number is connected with the height and more dry conditions. The variation is unprecedented: a nearly threefold range in copy number has been observed. A remarkable connection between the presence, within the *BARE-1* promoter of abscisic acid-response elements, and copy number variation suggesting that transposon proliferation may be stress-induced (Kalendar et al. 2000). Such increase in mobility at least partially might be adaptive producing rough material for new genes. Recent discoveries suggest a very high rate of gene creation by transposon capture and exon shuffling. In rice genome, for instance, *Mutator*-like DNA elements (MULEs) have been seen to carry fragments of genes and they were named “Pack-MULEs”. At least 5% of them are expressed and, by the criterion of expression these Pack-MULEs are already new genes (Bennetzen 2005). Another important factor possible influencing further diversification of *L. multiflorum* and *L. perenne* is the hybridisation with closely related genera e.g., *Festuca* genus. Whatever natural or artificial, it is able to mobilise transposons. In rice, the methylation pattern of transposon/retrotransposon sequences is dramatically changed after the introgression from *Zizania latifolia* (Dong et al. 2006).

And last but not least, despite all these changes are discrete and they might never entail to diversification of *L. multiflorum* and *L. perenne*, despite their diversification should be confirmed in wider phylogenetic studies involving the members of the whole genus, the present results suggest strongly that their future is to be distinct. Transposons, however slowly, but consistently reshaping genomes of all organisms. Why should they be silent in *Lolium*?

4.5. CONCLUSIONS

1. Genetic variation of *L. multiflorum* and *L. perenne* is high and typical of cross-pollinated species.
2. Surveys of the majority of marker analysed reveal nearly the same level of polymorphism in *L. multiflorum* and *L. perenne* as estimated from the overall proportion of polymorphic loci, mean number of alleles and the values of genetic diversity. The markers specific to a species are hardly found with the only exception of few bands revealed by SSAP.
3. The high differentiation of populations as measured by G_{ST} results from historical processes such as colonisation after the last glaciation and spread of agriculture as well as from intensive breeding. The populations in different regions may show different evolutionary trajectories.
4. No erosion of genetic resources in relation to breeding activities is observed in cultivars of *L. multiflorum* and *L. perenne*. However, the introgressions from closely related genera (*Festuca*) may be responsible for some distinctiveness of *L. multiflorum* cultivars.
5. High molecular similarity strongly supports the classification of *L. multiflorum* and *L. perenne* as a single species. Nevertheless, some mechanisms of "sudden speciation" can not be excluded prior to detail mapping studies.
6. Both DNA transposons and retrotransposons have been playing important role in *L. multiflorum* and *L. perenne* evolution. It is likely that *L. perenne* is more ancient than *L. multiflorum* but detailed phylogenesis can be clarified in a study involving the members of the whole genus and closely related genera.
7. The higher activity of transposons in *L. multiflorum* suggests further diversification of both species. The adaptation to more diverse environments and breeding activities will promote this process.

5. ORIGIN OF SEEDLING ROOT FLUORESCENCE IN *L. MULTIFLORUM* AND *L. PERENNE*

5.1. INTRODUCTION

Different uses of *L. multiflorum* and *L. perenne* have significant effect on selection criteria and result in phenotypic differences between their cultivars. *L. multiflorum* cultivars are primarily used for forage purposes while the majority of *L. perenne* is sold to turfgrass market. Therefore, Italian ryegrass cultivars often exhibit rapid vertical growth and lighter leaf colour than perennial ryegrass. The latter when used for permanent turf and lawns grows slowly, has dark and narrow leaves. If individuals of *L. multiflorum* get into *L. perenne* it results in undesirable turf quality. Hence, the main goal of breeders is to protect from contamination of one species by another. The overlapping range of morphological variation (Chapter 3) makes difficult separation of Italian ryegrass cultivars from perennial ones on easily scored characters especially in younger stages of development. The difficulties in species differentiation create a serious problem for breeders and seed certification agencies that remains to be solved. Methods to distinguish between annual and perennial types have long been sought.

Seedling root fluorescence as a phenotypic marker has been used to separate *L. multiflorum* from *L. perenne* since it discovery in 1929 by Gentner. Generally, seedling roots of annual ryegrass fluoresce when placed under ultraviolet light, and those of perennial ryegrass do not fluoresce (Niemyski and Budzyńska 1972; Barker and Warnke 1999). This brilliant blue glow of annual ryegrass roots is caused by an oxazole alkaloid which is leaked from the roots of developing seedlings onto a white substrate during germination. For the first time, the alkaloid was isolated from *L. multiflorum* roots in 1958 and named annuloline for "annual *Lolium*". The tentative empirical formula is $C_{20}H_{19}NO_4$ and there are three methoxyl groups (Figure 5.1). It is a week base, sparingly soluble in water and strongly fluorescent. As little as 0.01 μg of the alkaloid may be detected, when applied to a 2-mm diameter area on Whatman N°1 (Axelrod and Belzile 1958). A precursor of annuloline (Figure 5.1) is derived from ß-hydroxyphenylethylamine and a substituted cinnamic acid (Hardwick and Axelrod 1969). Seedling root fluorescence has been used widely as a supplement to morphological data but also, in the USA and Canada - as the only accepted method for detecting the presence of annual ryegrass in seed lots of perennial ryegrass (Niemyski and Budzyńska 1980). In 1953 the test was officially approved and adopted by the International Seed Testing Association (Jones 1983). In 1990, the Federal Seed Act rules for testing seeds allowed the seedling fluorescence test to be used as a cultivar descriptor in ryegrasses in the USA (Barker and Warnke 1999).

Application of the test, however, has some difficulties associated with a certain level of fluorescence in *L. perenne*. Generally, fluorescent *L. perenne* plants are regarded as a result of

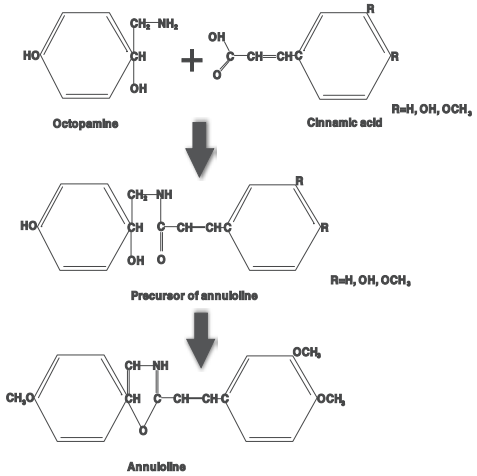


Figure 5.1. Biogenesis of annuloline (Hardwick and Axelrof 1969)

accidental introgression from *L. multiflorum* (Stuczyńska and Stuczyńska 1994). Consequently, fluorescence in perennial ryegrass is thought to be correlated with other features typical of *L. multiflorum* including leaf vernation, rapid growth, wide leaves and the others (Niemyski and Budzyńska 1984; Baker and Warnke 1999). Because tall, light green plants resembling *L. multiflorum* would detract from the otherwise pleasing appearance of perennial ryegrass turf, the perennial ryegrass breeders usually adopt the fluorescence test during vegetation and discard all fluorescent plants. Unfortunately, it turns out that few *L. perenne* seedlings always fluoresce. The difficulties in eliminating fluorescence inevitable lead to a hypothesis that it is also present in a gene pool of *L. perenne*. Due to the common gene pool of *L. multiflorum* and *L. perenne* (Chapter 4) lack of fluorescence in the latter would be strange. If fluorescence in *L. perenne* is introgressed from *L. multiflorum*, morphology of fluorescent plants will be slightly different in comparison with nonfluorescent plants. Conversely, florescent and nonfluorescent plants will be alike as long as fluorescence is of intrinsic origin. Thus, the objective of this research was to clarify the origin of fluorescence in *L. perenne* by comparing the morphology of fluorescent and nonfluorescent plants. Having in mind that the level of fluorescence in *L. perenne* cultivars can be understated artificially due to breeding strategies, inclusion of wild ecotypes in addition to cultivars was a crucial point of these analyses. It is also interesting if seminal root fluorescence is a characteristic feature of *L. multiflorum* or the genes encoding this character are present in the gene pool of the whole genus *Lolium*. Thus, for the first time, the other representatives of the genus *Lolium* i.e., *L. loliaceum*, *L. remotum*, *L. temulentum, L. persicum* and *L. rigidum* were included in the analysis as well.

Another difficulty in applying seedling root fluorescence is associated with environmental variation and test conditions not being standardized. Although the International Seed Testing Association and the Association of Official Seed Analysts Rules specify that the test goes the entire 14 days (USDA 2006) after germination, some authors recommend 18 up

to 28 days so that every seed is given a chance to express any fluorescence trait (Niemyski and Budzynska 1972). Also, there is quite a broad range of accepted temperature (20-30°C) and light (darkness or photoperiod) regime. Moreover, in the original protocol seeds are sown directly into a germination box or arranged on glass plates. However, this procedure has at least two limitations. Firstly, there is unequal number of germinated seeds in each replication; secondly, there is a risk that lack of fluorescence can result from slower development of some seedlings. Therefore, in the preliminary experiments, the original procedure elaborated by Niemyski and Budzyńska (1972) was modified as well as temperature and light conditions were optimized.

The difficulties in seedling root fluorescence have prompted the search for alternative tests. This has resulted in the identification of several enzymatic markers (Nakamura 1979; Payne et al. 1980; Warnke et al. 2002) but they were based on differences in allele frequencies and thus, none have been developed into a suitable alternative to fluorescence. Regardless how Italian and perennial ryegrasses are classified, they are used for different purposes and any reliable technique that will enable early detection of seed contamination of one type by another certainly will safe time and money. Although seedling root fluorescence is not exact, it has been successfully used for many years. Nevertheless, it would be better to replace it by the method more stable and less prone to human subjectivity. Therefore, the study was also undertaken to identify robust DNA markers that distinguish fluorescent from nonfluorescent forms in *L. multiflorum* and *L. perenne*.

5.2. MATERIAL AND METHODS

5.2.1. Optimisation of seedling root fluorescence test

Two *L. multiflorum* cultivars, Asso and Bartissimo, were used in all optimisation experiments. The seedling root fluorescence test (the Gentner's test) was primarily based on the procedure described by Niemyski and Budzyńska (1972). The major modification of this procedure laid in germination of seeds on white filter paper in Petri dishes instead of glass plates. Germination in Petri dishes was conducted at 24°C in darkness for three, five and six days. Then the seeds were checked for the presence of any sign of fluorescence and 20 seedlings of equal size were transferred onto glass plates for seedling root fluorescence tests. The following parameters were checked: the duration of the germination, temperature during the test, influence of light, duration of the test. Each test was conducted at three different dates and each run had three replications. Seedling root fluorescence was visualized at 300 nm using UV transilluminator. The data were pooled for the three runs. The level of fluorescence was estimated as the percentage of fluorescent seedlings in relation to a total number of normal seedlings followed standard protocols (USDA 2006). The optimised protocol is described in Annex 13.2.

5.2.2. Level of seedling root fluorescence

To compare the level of seedling root fluorescence in annual and perennial ryegrass in total 17 *L. multiflorum* and 26 *L. perenne* populations were used including cultivars and wild ecotypes. Additionally, samples of the other *Lolium* species were analysed i.e., *L. loliaceum*, *L. persicum*, *L. remotum*, *L. temulentum* and *L. rigidum*. All populations are described in Annex 13.1. About 300 seeds of each *L. multiflorum* and *L. perenne* population were germinated at 24°C for six days in order to obtain 240 equally developed seedlings. In total 20 seedlings were transferred onto a single glass plate. In case of *L. loliaceum*, *L. persicum*, *L. remotum*, *L. temulentum* and *L. rigidum*, due to low number of seeds, only 100 seeds were used and about 80-90 seedlings were analysed. After 14 days seedling roots were scored for the presence (+) and absence (-) of fluorescence under UV light (Figure 5.2). All plants with fluorescent roots (+) and all plants with nonfluorescent roots (-) were rescued for further analyses. Each population was analysed in four runs with three randomly arranged replications in each run. The level of fluorescence was calculated in relation to a total number of normal seedlings. Data were pooled for the four runs and they were analysed using one-factor ANOVA with LSD test in STATISTICA 7.1. The squared Mahalanobis distance was estimated between all species from the genus *Lolium* based on fluorescence level. The Euclidean distance and Ward's method were applied for clustering.

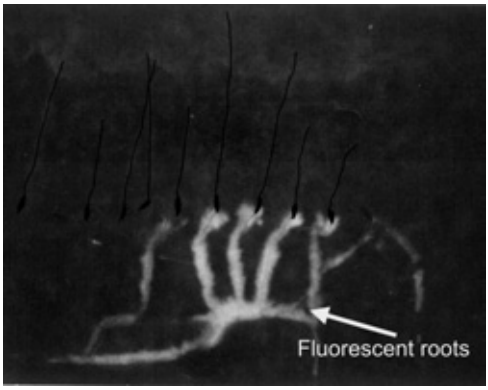


Figure 5.2. Seedling root fluorescence test

5.2.3. Development of populations for morphological analyses and analysed characters

Only these populations were analysed, in which both fluorescent and nonfluorescent seedlings were found in sufficient amount to set up a field experiment. In total nine *L. multiflorum* including seven cultivars and two ecotypes and nine *L. perenne* populations including five cultivars and four ecotypes were used in morphological analyses. After estimation of fluorescence of seminal roots the plants were transferred into pots and as soon as they developed into multitillered plants, all plants were divided into 9-10 ramets and transplanted to the experimental field in April and early May 2002. A randomized complete block design with three replications was used. Each block consisted of three ramets as replicates

of each genotype. Plants were sown randomly in each replication. From 10 to 15 fluorescent genotypes and 10-15 nonfluorescent genotypes were analysed per a population. Plants were spaced on 50 cm by 50 cm grids.

Seedling characters (length of leaf and root) were scored together with seedling root fluorescence determination i.e., on the 14th day after germination. Adult vegetative characters were scored during two crops in July and September 2003. For each plant, three average-size basal leaves were harvested and protected with a paper envelope. The leaf length and width were measured next day and the data were averaged for each plant. Flowering characters were studied only in *L. multiflorum* because not enough *L. perenne* plants flowered in the 1st year of cultivation. The plant height was determined before cut. The three well developed spikes together with flag leaves were collected from each plant, placed in a paper bag and used for spike, spikelets and floret character measurements. The areas of vegetative and flag leaves were counted by multiplication of the leaf length by leaf width.

Analysis of variance with LSD test was used to examine differences between fluorescent and nonfluorescent plants. Correlation coefficients were estimated for each character to find out relationships between fluorescence and morphological features. Data were analysed using STATISTICA 7.1 software.

5.2.4. Identification of DNA markers associated with seedling root fluorescence

A population of *L. multiflorum*, Bartissimo and a population of *L. perenne*, Argona were chosen to search for putative DNA markers correlated with seedling root fluorescence. A variant of bulked segregant analysis (BSA) adopted for population of outbreeding species was used as the basic method of screening. Two bulks per each population were made; the first consisted of ten fluorescent plants and the second of ten nonfluorescent plants. The DNA was extracted as described in Annex 13.4. Two bulks were assumed to be dissimilar in the genomic region around fluorescence genes but seemingly heterozygous and monomorphic at all over regions. Markers adjacent to the fluorescence gene(s) should be in linkage disequilibrium. As the distance increases, more recombinants should be present in each bulk. Unlinked markers or markers in linkage equilibrium were expected to be randomly distributed across bulks (Lynch and Walsh 1998). DNA from each bulk was screened en mass with 54 RAPD, three ISJ primers and a pair of katG10 primers (Annex 13.5) as described in Annex 13.8, 13.9 and 13.13. Generally dominant markers such as RAPDs and ISJs enable to identify markers in 2-15-centimorgan (cM) distance from a target gene (Michelmore et al. 1991). To confirm the relationship between fluorescence and DNA markers five individuals from fluorescent and nonfluorescent bulks were evaluated with candidate primers which showed the same type of polymorphism between the bulks in both species. Candidate bands that revealed consistent differences between fluorescent and nonfluorescent plants were considered potential markers associated with fluorescence. These markers were validated on fluorescent and nonfluorescent bulks from three cultivars of *L. multiflorum*, namely Atalja, Asso and Crema and two cultivars of *L. perenne*, namely Rela and Solen.

5.3. RESULTS

5.3.1. Optimisation of seedling root fluorescence test

The major modification of seedling root fluorescence test i.e., the germination of seeds in Petri dishes instead of glass plates proved to be very advantageous because all seedlings were in the same stage of development after the 18-day long test and fluorescence could be reliably read. The highest level of fluorescence was observed after six days of germination (Table 5.1A) and this period was applied in all subsequent experiments. The durations of germination could not be longer than seven days since after that time roots started to fluoresce.

The level of fluorescence depended also on temperature and the highest number of fluorescent seedlings were observed at 27°C (Table 5.1B). Noteworthy, the light exposure reduced strongly the level of fluorescence even though the boxes, where glass plates with seedlings were put, were wrapped in aluminum foil (Table 5.1C). This is probably due to decomposition of annuloline by light.

From the preliminary experiments it is obvious that the duration of seedling root fluorescence tests should not exceed 14 days (Table 5.1.D) what is in agreement with the Association of Official Seed Analysts Rules (IOA 2006) but in contrast to Niemyski and Budzyńska (1972) recommendations and Polish standards PN-79/R-65950. The prolonged period of seedling incubation resulted in loss of fluorescence mainly due to root death. This also caused difficulties in seedling rescue.

Table 5.1. Optimisation of the seminal root fluorescence test

Cultivar	Percentage of seedling root fluorescence		
A. Influence of the germination lenght			
	The duration of the germination in days		
	3 days	**5 days**	**6 days**
Bartissimo	44.33	50.00	57.00
Asso	36.66	61.20	65.00
Other parameters during the test: 27°C, darkness, test duration – 18 days			
B. Influence of temperature			
	Temperature		
	24°C	**27°C**	**30°C**
Bartissimo	41.22	56.00	37.12
Asso	45.06	60.00	31.22
Other parameters during the test: 6 days of germination, darkness, test duration – 18 days			
C. Influence of light			
	Light	**Darkness**	
Bartissimo	10.00	58.00	
Asso	20.00	62.00	
Other parameters during the test: 6 days of germination, 27°C, test duration – 18 days			
D. The duriation of the test in days			
	14	**18**	
Bartissimo	54.00	48.00	
Asso	55.00	53.00	
Other parameters during the test: 6 days of germination, 27°C, darkness			

5.3.2. Level of seedling root fluorescence in the genus *Lolium*

Unsurprisingly, the level of seedling root fluorescence was significantly higher in *L. multiflorum* than in *L. perenne* (Table 5.2). No differences were recorded between cultivars and ecotypes. Similarly, the majority of populations within a species did not differ significantly. The percentage of fluorescent roots in *L. multiflorum* ranged from 74% to 100%, however the absolute fluorescence (100%) was observed in only 24% of populations. Interestingly, it was only ecotypes. The lowest level of fluorescence was recorded in Crema. It was twofold lower than in the other populations. The level of seedling root fluorescence in *L. perenne* was

Table 5.2. Level of fluorescence in *L. multiflorum* and *L. perenne* genotypes

Species	Cultivar/ecotype	Germinated seeds [%]			Frequency of fluorescent seeds ±SD		
L. multiflorum	**Asso**	76.7	**Cultivars** 63.2	**Species 65.1**	0.99±0.02^{ab}	**Cultivars** 0.81±0.15^{a}	**Species 0.87±0.15^{a}**
	Atalja	68.9			0.79±0.09^{bc}		
	Bartolini	84.3			0.83±0.01^{abc}		
	Bartissimo	50.1			0.75±0.23^{cd}		
	Crema	57.7			0.46±0.05^{e}		
	Ligrande	75.6			0.96±0.06^{abc}		
	Limulta	68.9			0.89±0.07^{abc}		
	Miyukiaoba	50.6			0.78±0.21^{bc}		
	Nagamamikari	35.0			0.78±0.39^{bc}		
	Tenor	95.8			0.98±0.02^{ab}		
	Waseaoba	31.7			0.74±0.25^{cde}		
	Barball	76.2	**Ecotypes** 72.2		1.00±0.00^{a}	**Ecotypes** 0.97±0.07^{a}	
	German ecotype	41.7			0.97±0.04^{abc}		
	Guliamo	68.5			1.00±0.00^{a}		
	Italian ecotype	100.0			1.00±0.00^{a}		
	Spanish ecotype	74.4			0.83±0.19^{abc}		
	Variamo	72.3			1.00±0.00^{a}		
L. perenne	**Aberoscar**	46.7	**Cultivars** 86.9	**Species 79.7**	**0.56±0.10^{de}**	**Cultivars** 0.07±0.16^{b}	**Species 0.08±0.17^{b}**
	Argona	90.8			0.19±0.14^{f}		
	Ba012	100.0			0.00±0.00^{f}		
	Kyosato	84.7			0.00±0.00^{f}		
	Lisuna	100.0			0.00±0.00^{f}		
	Magella	90.0			0.05±0.06^{f}		
	Merganda	85.5			0.01±0.02^{f}		
	Numan	79.0			0.00±0.00^{f}		
	Pamir	87.2			0.00±0.00^{f}		
	Portstewart	100.0			0.00±0.00^{f}		
	Rela	84.5			0.03±0.03^{f}		
	Solen	95.0			0.02±0.00^{f}		
	704	42.0	**Ecotypes** 73.5		0.07±0.02^{f}	**Ecotypes** 0,08±0.19^{b}	
	708	98.0			0.00±0.00^{f}		
	NGB4262	58.9			0.02±0.03^{f}		
	NGB4264	100.0			0.08±0.11^{f}		
	NGB5030	51.7			**0.76±0.13^{bcd}**		
	NGB5031	35.0			0.00±0.00^{f}		
	NGB5036	100.0			0.04±0.00^{f}		
	NGB10793	100.0			0.00±0.00^{f}		
	NGB10795	95.0			0.00±0.00^{f}		
	NGB10809	67.1			0.05±0.00^{f}		
	NGB10815	60.0			0.05±0.01^{f}		
	Tatras	58.4			0.03±0.01^{f}		
	Hungarian	71.8			0.06±0.04^{f}		
	New Zealand	91.2			0.01±0.02^{f}		

Different letters mean significant differences at P=0.05 for LSD test

very low and ranged from 0% to 19%. In about 38% of populations no fluorescent seedling was found. Fluorescent seeds were observed with the same frequency in cultivars and wild ecotypes although in the former 50% of populations did not show any fluorescence (0%) while in the latter such populations constituted only 29%. There were also populations, in which the level of fluorescence was high and comparable to *L. multiflorum*. Surprisingly high percentage of fluorescent roots was observed in *L. perenne* cultivar, Aberoscar (56%) and in Scandinavian ecotype NGB5030 (76%).

Examination of the level of fluorescence in the other *Lolium*s showed that this feature is not specific to *L. multiflorum* but it is present in the whole genus (Table 5.3). The genus could be divided into three species groups depending on the level of fluorescence (Figure 5.3). The first group consisted of allogamous *L. rigidum* and *L. multiflorum*, in which the number of fluorescent seeds was the highest, 87% and 90%, respectively. The three autogamous species, *L. loliaceum*, *L. remotum* and *L. temulentum* with about half fluorescent seedling roots (48%-53%) formed the second group. The third group consisted of both autogamous and allogamous species with low and no fluorescence. *L. persicum* (0%) and *L. perenne* (8%) belonged to this group.

Table 5.3. Level of fluorescence in seven *Lolium* species

Species	Germinated seeds [%]	Frequency of fluorescent seeds ±SD
Lolium loliaceum	95.0	0.53 ± 0.04^{ab}
Lolium persicum	57.7	0.00 ± 0.00^{c}
Lolium remotum	74.0	0.48 ± 0.03^{ab}
Lolium temulentum	79.8	0.53 ± 0.36^{ab}
Lolium multiflorum	65.1	0.87 ± 0.17^{a}
Lolium perenne	79.7	0.08 ± 0.17^{bc}
Lolium rigidum	78.8	0.90 ± 0.02^{a}

Different letters mean significant differences at P=0.05 for LSD test

5.3.3. Correlation between seedling root fluorescence and morphological characters

The majority of vegetative traits were not correlated with seedling root fluorescence both in *L. multiflorum* and *L. perenne* (Table 5.4). Similarly, the variation in generative characters analysed only in *L. multiflorum*, did not correspond to the differences in fluorescence (Table 5.5). The mean values in fluorescent and nonfluorescent plants were mostly alike (Table 5.6). The only exceptions were seedling leaves and roots in *L. multiflorum*, for which significant correlation coefficients were observed. To examine the nature of this correlation, the analysis was done separately for cultivars and ecotypes. It became obvious that faster growth of fluorescent seedling in *L. multiflorum* was attributable to cultivars of this species (Table 5.7). Fluorescent seedlings had 23% longer leaves and 18% longer roots than nonfluorescent ones (Table 5.6). No differences in seedling leaves and roots were found in ecotypes. However, in ecotypes, fluorescent plants had wider basal leaves although this character was not transformed into the significant correlation coefficient. Somehow different relationships were observed in *L. perenne* cultivars. In this case nonfluorescent plants had higher green weight, but againit was not transformed into the correlation coefficient.

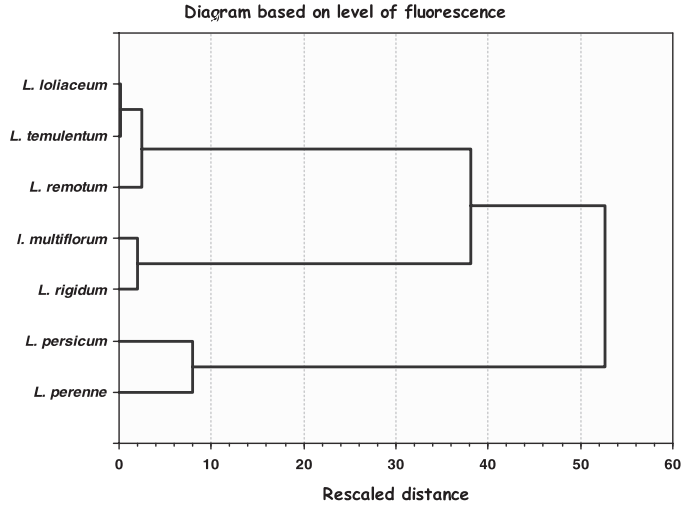


Figure 5.3. Phenogram based on the squared Mahalanobis distance between seven *Lolium* species, clustered using Ward's method

Table 5.4. Correlation coefficients between seedling root fluorescence and vegetative characters in *L. multiflorum* and *L. perenne*

Character	*L. multiflorum*	*L. perenne*
Seedling leaf length	0.312**	0.071
Seedling root length	0.288*	0.073
Basal leaf length	-0.072	0.132
Basal leaf width	0.041	0.135
Basal leaf area	-0.028	0.083
Green weight	-0.081	-0.289
Dry weight	-0.042	-0.239

*Significant at P=0.05, **Significant at P=0.01

Table 5.5. Correlation coefficients between seedling root fluorescence and generative characters in *L. multiflorum*

Character	*L. multiflorum*
Height at ear emergence	-0.212
Spike length	0.044
Spikelet length	-0.109
Spikelet number	0.043
Floret number	-0.079
Flag leaf length	-0.202
Flag leaf width	0.077
Flag leaf area	-0.158

Table 5.6. Comparison of seedling and vegetative characters in fluorescent and nonfluorescent plants of *L. multiflorum* and *L. perenne*

Species	F/NF*	Seedling leaf length [cm]±SD	Seedling root length [cm]±SD	Basal leaf length [cm]±SD	Basal leaf width [cm]±SD	Basal leaf area [m^2]±SD	Green weight [g]±SD	Dry weight [g]±SD
L. multiflorum	**F**	**6.5±1.7**	**3.9±0.8**	30.4±6.9	0.46±0.11	14.5±5.3	108.5±57.0	29.6±19.9
	NF	**5.4±1.7**	**3.4±0.9**	31.4±5.9	0.45±0.08	14.3±4.4	118.9±72.3	31.5±25.1
cultivars	F	**6.9±1.5**	**4.0±0.8**	29.9±7.5	0.45±0.12	14.1±5.8	119.4±58.6	32.7±21.2
	NF	**5.6±1.7**	**3.4±1.0**	32.3±5.1	0.47±0.09	15.1±4.1	133.0±73.6	35.8±26.3
ecotypes	F	4.2±0.9	3.5±0.6	32.3±3.3	**0.50±0.06**	16.1±2.0	65.2±16.3	17.4±3.6
	NF	4.2±0.9	3.2±0.5	27.6±7.9	**0.39±0.04**	11.0±4.1	62.8±23.4	14.3±4.4
L. perenne	**F**	5.7±1.6	3.8±1.2	34.4±8.6	0.43±0.11	14.7±5.3	**149.1±22.9**	41.5±28.3
	NF	5.5±1.3	3.8±1.1	32.7±4.9	0.40±0.07	13.4±3.9	**208.5±124.5**	57.7±39.7
cultivars	F	5.8±1.8	3.6±1.3	35.9±9.6	0.45±0.11	16.2±5.4	**166.5±85.1**	48.2±29.9
	NF	5.7±1.4	3.6±1.1	33.7±7.3	0.43±0.02	14.6±3.9	**239.4±125.8**	68.3±40.6
ecotypes	F	5.3±0.5	4.2±1.0	29.8±1.0	0.35±0.01	10.4±0.6	96.9±10.0	21.3±1.00
	NF	4.9±0.9	3.7±0.9	29.4±1.0	0.33±0.01	9.7±0.6	115.6±25.2	25.8±1.00

***F –** fluorescent plants, **NF –** nonfluorescent plants
Bold – significant differences between fluorescent and nonfluorescent plants at P=0.05 for LSD test

Table 5.7. Correlation coefficients between seedling root fluorescence and seedling characters in cultivars and ecotypes of *L. multiflorum* and *L. perenne*

Character	*L. multiflorum*		*L. perenne*	
	Cultivars	Ecotypes	Cultivars	Ecotypes
Seedling leaf length	0.379**	0.030	0.025	0.254
Seedling root length	0.303*	0.236	0.029	0.254

*Significant at P=0.05, **Significant at P=0.01

5.3.4. Identification of DNA markers associated with seedling root fluorescence

In total 650 reliable read amplification products were identified in two pairs of bulks derived from *L. multiflorum* and *L. perenne*. More than half bands (55%) were polymorphic between all bulks. In each species about one fourth of all bands detected polymorphism between fluorescent and nonfluorescent bulks (Table 5.8). The majority of markers revealed differences only in one pair of bulks but not in the other. Only 10% of all bands were polymorphic between fluorescent and nonfluorescent bulks in both species. Among them about 5-6% were associated in a coupling linkage phase with fluorescence amplifying the polymorphic fragment only in fluorescent plants (Table 5.9). The other 4-6% fragments were amplified only in nonfluorescent plants and thus, were associated in a repulsion linkage phase. However, the majority of markers were in different linkage phases in each species. The same linkage phase in *L. multiflorum* and *L. perenne* was observed for only a slightly more than 2% of bands. In the case of ten bands (1.5%), fluorescent bulks both from *L. multiflorum* and *L. perenne* had a band (coupling phase) while in the case of five bands (0.8%) fluorescent bulks did not have any band (repulsion phase). To confirm the association of these 15 bands with fluorescence individual plants were analysed from each bulk. For three markers i.e., OPA11-6, OPA20-3 and OPB10-19, the similar pattern as in bulks was consistently shown

by individual fluorescent and nonfluorescent plants (Table 5.10). The OPA11-6 and OPA20-3 were linked with fluorescence in coupling phase whereas the OPB10-19 in a repulsion phase (Figure 5.4). These three fragments were considered to be putative markers for fluorescence and were successfully validated for their specificity on three *L. multiflorum* and two *L. perenne* cultivars (Table 5.11). Two markers, OPA20-3 and OPB10-19 were confirmed in both species whereas the OPA11-6 marker was confirmed only in *L. multiflorum*. In Asso, the OPB10-19 was probably in different linkage phase than in the other cultivars. None of these markers were successful in discrimination of fluorescent and nonfluorescent plants in Atalja

Table 5.8. Polymorphic bands beetwen fluorescent and nonfluorescent bulks of *L. multiflorum* and *L. perenne*

Species	Bands polymorphic between bulks in each species		Bands polymorphic between bulks in both species	
	N°	%	N°	%
L. multiflorum	166	25.5	68	10.5
L. perenne	152	23.4		

Table 5.9. Screening of fluorescent and nonfluorescent bulks of *L. multiflorum* and *L. perenne* with DNA markers

Species	Bands associated in a coupling phase with fluorescence		Bands associated in a repulsion phase with fluorescence	
	N°	%	N°	%
L. multiflorum	41	6.3	27	4.2
L. perenne	32	4.9	36	5.6
L. multiflorum + L. perenne	10	1.5	5	0.7

Table 5.10. Individual plant data of bullk specific RAPD fragments for fluorescent and nonfluorescent individuals of *L. multiflorum* and *L. perenne*

Marker	*L. multiflorum*												*L. perenne*											
	Bulks		Individual plants										Bulks		Individual plants									
	F	NF	F	F	F	F	F	NF	NF	NF	NF	NF	F	NF	F	F	F	F	F	NF	NF	NF	NF	NF
OPD05-11	**1**	**0**	1	1	1	1	1	0	0	1	0	0	**1**	**0**	0	0	1	1	1	0	0	0	0	0
OPA11-6	**1**	**0**	1	1	1	1	1	0	0	0	0	0	**1**	**0**	1	1	1	1	1	0	0	0	0	0
OPA20-3	**1**	**0**	1	1	1	1	1	0	0	0	0	0	**1**	**0**	1	1	1	1	1	0	0	0	0	0
OPB02-9	**1**	**0**	1	1	1	0	1	0	0	0	0	0	**1**	**0**	1	1	0	0	1	0	0	0	0	1
OPB03-1	**1**	**0**	1	1	1	1	1	0	0	0	0	1	**1**	**0**	1	1	1	1	0	0	0	0	0	0
OPB06-12	**1**	**0**	1	1	0	0	1	0	0	1	0	0	**1**	**0**	0	1	1	1	1	0	0	0	0	0
OPB10-12	**1**	**0**	1	1	1	1	1	0	0	0	1	0	**1**	**0**	1	1	1	0	1	0	0	0	0	0
OPB11-9	**1**	**0**	1	1	1	1	1	1	0	0	0	0	**1**	**0**	1	1	1	1	0	1	0	0	0	0
OPB11-12	**1**	**0**	1	1	0	1	0	0	0	0	0	0	**1**	**0**	1	1	1	1	1	0	0	1	0	0
OPB11-13	**1**	**0**	0	0	1	1	1	0	0	0	0	0	**1**	**0**	1	1	1	1	1	1	0	0	0	0
OPA12-10	**0**	**1**	0	0	0	0	0	1	1	1	0	0	**0**	**1**	0	0	0	0	1	1	1	1	0	0
OPB07-11	**0**	**1**	0	0	0	0	0	1	1	0	1	1	**0**	**1**	0	0	1	0	0	1	1	1	1	1
OPB08-19	**0**	**1**	0	0	0	0	1	1	1	1	1	1	**0**	**1**	0	0	0	0	0	0	1	1	1	1
OPB08-21	**0**	**1**	0	0	0	0	0	1	0	1	1	1	**0**	**1**	1	0	0	0	0	1	1	1	1	1
OPB10-19	**0**	**1**	0	0	0	0	0	1	1	1	1	1	**0**	**1**	0	0	0	0	0	1	1	1	1	1

F – fluorescent plants, NF – nonfluorescent plants
1 – indicates amplification of the band, 0 – indicates the absence of amplification

Table 5.11. Validation of the RAPD markers associated with fluorescence on *L. multiflorum* and *L. perenne* cultivars

Marker	*L. multiflorum*						*L. perenne*			
	Asso		Atalja		Crema		Rela		Solen	
	F	NF	F	NF	F	NF	F	NF	F	NF
OPA11-6	1	0	1	1	1	0	1	1	1	1
OPA20-3	0	0	1	1	1	0	1	0	1	0
OPB10-19	1	0	1	1	0	1	1	1	0	1

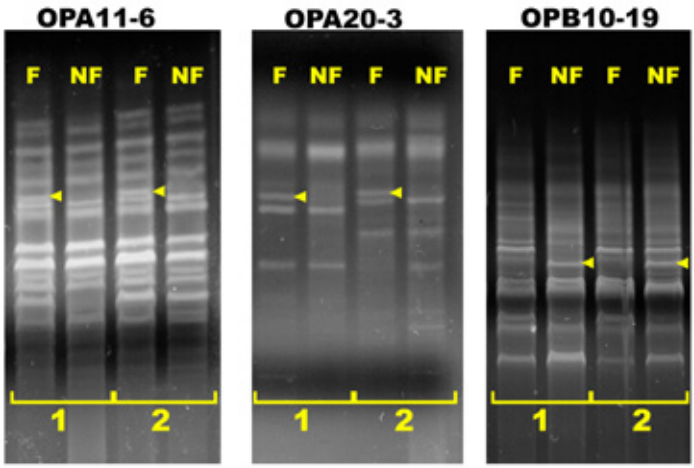


Figure 5.4. RAPD markers associated with fluorescence
(1. *L. multiflorum*, 2. *L. perenne*)

5.4. DISCUSSION

Tests or markers that enable to distinguish between closely related species are extremely helpful in breeding, especially if species are very difficult to classify solely on the basis of their morphological characters. Such tests need to be fast, easy and inexpensive. The present data provide an empirical example that seedling root fluorescence works fairly well in the majority of populations and thus, has some power in applications such as seed contamination assessment. As expected the level of fluorescence is high in *L. multiflorum* and very low in *L. perenne*. However, the test is not exact and perennial ryegrass populations with extremely high fluorescence are observed e.g., cultivar Aberoscar and Scandinavian ecotype NGB5030 as well as nonfluorescent Italian ryegrass populations do exist (Floyd and Barker 2002). Variation across generations, years and locations has been also noted (Stuczynska and Stuczynska 1994). Unfortunately, the reasons of this variability have not been well understood and therefore, fluorescence should not be used as infallible guides to classification of questionable seeds. On the other hand, the debate about the test application has come to a standstill presumably because the reason underlying fluorescence in *L. perenne* has been misunderstood.

For any species specific test, compared genomes have to be diverse enough to create distinct markers for each species. A noteworthy example of how genetic markers can distin-

guish closely related species involves *Agrostis capillaris* and *A. palustris* (Scheef et al. 2003). Surveys of RAPD markers revealed two bands that correctly classified 17 cultivars. However, *L. multiflorum* and *L. perenne* have the same genome constitution and even a high-throughput technology such as SSAP is not sufficient enough to identify specific markers for a wide range of cultivars revealing only few unique bands (Chapter 4). This sobering example of how difficult is to find DNA markers diversifying *L. multiflorum* and *L. perenne* demonstrates, that it can not be expected for biochemical characters like seedling root fluorescence. In this instance, an approach similar in concept to traditional labelling of cultivars within a species would rather be applied. The searches for "virtual" species specific markers should be replaced by searches for markers linked to characters important for turf quality.

Conversely, application of seedling root fluorescence assumes that *L. multiflorum* and *L. perenne* are different biological species and therefore, they differ in many characters, fluorescence is just one of them. The seedling root fluorescence in *L. perenne* is considered to be introduced from *L. multiflorum* (Floyd and Barker 2002). If both species grow in vicinity the level of fluorescence in perennial ryegrass is expected to increase across generations as a result of spontaneous hybridisation. This phenomenon was illustrated in perennial and hybrid ryegrass cultivars, for which the percentage of fluorescent roots was increasing from 74% to 100% during three years of studies (Niemyski and Budzyńska 1984). However, this example may be misrepresentative firstly, because perennial ryegrass cultivar had unusually high level of fluorescence, and secondly, fluorescence was not track in the same population harvested, multiplied, and resown at the same location. Yet, generation response strongly depends on environmental conditions. Little or no change of seedling root fluorescence is observed in one location while in the other fluorescence increases in next generations (Floyd and Barker 2002). *L. multiflorum* pollen is believed to be a source of contamination and therefore, the cereal grain isolation barriers during growth and the elimination of fluorescent seeds are recommended. This strategy is only partially successful. Indeed, half perennial ryegrass cultivars studied have not exhibited any fluorescence (0%) while only 29% of all ecotypes. But the average level of fluorescence in cultivars and ecotypes is mostly the same. Perhaps it is not possible to eliminate fluorescence from perennial ryegrass cultivars and although with low frequency it will always be present in the gene pool of this species.

A central difficulty in interpreting fluorescence in *L. perenne* as a result of introgression lies in understanding the degree to which foreign pollen contamination may account for the presence of fluorescence in populations isolated from *L. multiflorum*. Scandinavian ecotypes or the ecotype from the Tatras were collected from areas outside the distribution range of Italian ryegrass, yet fluorescence is present in about 8% of plants. It seems unlikely that it is a result of contamination by Italian ryegrass pollen. Copeland and Hardin (1970) showed that perennial ryegrass plants are fertilised primarily by pollen from neighbouring plants. They found little contaminant Italian ryegrass pollen in perennial ryegrass beyond 6 m and no evidence of contamination beyond 12 m. Similarly, Floyd and Barker (2002) calculated that from 11 to 42% immigration would be needed to account for about 5% increase of fluorescence across generations. They concluded that this level of contamination is highly unlikely. From that perspective, it should be assumed that fluorescence in *L. perenne* ecotypes is rather intrinsic origin that extrinsic i.e., from *L. multiflorum*.

A second potential difficulty arising from the “introgression concept” is lack of correlation between fluorescence and morphological characters observed in the present work. Likewise, Nyquist (1963) indicates that the fluorescence trait does not influence growth habit and it is not associated with any trait that conditions annuality. This is opposite to an important generality that fluorescence and characters typical of *L. multiflorum* such as faster growth, higher yield, and lower winterhardiness are often correlated (Niemyski and Budzynska 1984; Stuczynska and Stuczynska 1994). The absence of significant *L. multiflorum* characteristics in fluorescent plants of *L. perenne* suggests that fluorescence does not result from contamination by Italian ryegrass pollen. Thus, it is conceivable that fluorescent plants are neither introgressive forms nor *L. multiflorum*. It should be noted however, that the present data do not exclude the possibility of linkage between fluorescence and QTLs responsible for morphological characters. Because cultivars and ecotypes were analysed they simply could be homozygous at QTLs encoding analysed traits. The existence of linkage can only be checked in a mapping population, in which QTLs and fluorescence segregate.

The overall picture from the present analyses is that genes encoding fluorescence are present in the gene pool of both *L. multiflorum* and *L. perenne*. One or two major dominant genes control seedling root fluorescence (Barker and Warnke 1999). The genotypic data provided by DNA markers may also suggest that more genes are responsible for this character. Three RAPD markers linked to seedling root fluorescence have been found in initial screening of bulk samples from *L. multiflorum* and *L. perenne*. Although this association may be accidental there is minimal chance that regions unlinked to the target region will differ between the bulked samples of many individuals (Michelmore et al. 1991). Nevertheless, none of these three RAPD markers is valid for all *L. multiflorum* and *L. perenne* cultivars. This observation may simply reflect the likehood that fluorescence in some cultivars is derived from a gene different than in initial bulks or from a combination of different genes. Like RAPD markers in the present work, specific markers designed on polymorphism within *Pm3* locus coding powdery mildew resistance in wheat, fail to amplify a specific band in several resistant cultivars. In these cultivars resistance could be provided by other *Pm* genes (Tommasini et al. 2006).

The pathway leading to annuloline, as for many other secondary compounds is quite simple and includes the 2-step conversion of ß-hydroxyphenylethylamine and substituted cinnamic acid into the fluorescent product (Figure 5.1). Presumably each step is catalysed by a single enzyme and each enzyme is controlled by a single gene. Assuming at least two complementary genes, fluorescence appears only when dominant alleles at both loci are present together. Thus, lack of fluorescence would be due to recessive point mutations leading to loss of enzymatic activity in either one of two genes. In fact, nonfluorescent plants are a mixture of different nonallelic and allelic mutants. If two nonallelic mutants hybridize fluorescence will be restored in offspring as a result of complementation of dominant alleles at each locus controlling the annuloline biosynthesis pathway. The frequency of fluorescent offspring will depend on homo - or heterozygous stage of mutants. Only if both mutants are homozygous the whole offspring will have fluorescent roots. The difference between *L. multiflorum* and *L. perenne* is in frequency of dominant and recessive alleles. The high level of fluorescence in *L. multiflorum* suggests that dominant alleles of both genes are very frequent. Conse-

quently, the situation that dominant alleles at each locus occur in a single plant is very common. Yet, there are still about 20% of nonfluorescent plants probably lacking dominant alleles at least at one of loci as a result of segregation and recombination. By contrary, dominant alleles are very rare in *L. perenne* and it is highly unlikely that they would meet in a single plant. Considering that the whole *L. perenne* population consists from nonfluorescent plants, the probability that two plants with dominant alleles at different loci would cross is only about 8%. Although the gene interaction described needs to be confirmed in crossing experiments, it suits very well to observed frequencies of fluorescence in *L. multiflorum* and *L. perenne* and explains why the elimination of fluorescence from *L. perenne* is almost impossible. Under the hypothesis of complementary genes it is clear that fluorescent plants in *L. perenne* have not arisen from contamination by *L. multiflorum* pollen and subsequent introgression. Therefore, they do not possess any morphological characters typical of *L. multiflorum*. Their emergence would rather be expected as an accomplishment of Mendelian laws on a population level.

For many years fluorescence has been regarded as a diagnostic character typical of *L. multiflorum*. The present findings exemplify how one widely held belief might be incorrect if genealogical context is taken into account. One fortunate consequence of the analysis of the whole genus *Lolium* is that fluorescence is rather a rule than exception. Point mutations can cause loss of ability to fluoresce and it seems that they have arisen independently in each species. Therefore, in each species some nonfluorescent plants can be found. Somehow lower frequency of fluorescent plants in autogamous species (*L. loliaceum*, *L. remotum* and *L. temulentum*) in comparison with allogamous ones (*L. multiflorum*, *L. rigidum*) can be connected with breeding system. In inbreeding species, fixation of new alleles is faster than in outbreeding, *L. perenne*. Another aspect of concern is to explain the differences between *L. multiflorum* and *L. perenne* in ability to produce annuloline from evolutionary pointof view.

One possible explanation involves different endophytes that grow in or on the roots of grasses and that are disseminated through seeds. The ability to secrete alkaloids into the rhizosphere by plant roots is a significant carbon cost to the plant however, it plays symbiotic or defensive roles (Walker et al. 2003). Grasses produce alkaloids less frequently than most other plants and therefore, they have developed in their evolution associations with fungi that produce a range of alkaloids or stimulate the host grass to synthesize alkaloids (Malinowski and Belesky 2000). Endophyte-infected grasses express a wide range of adaptation to biotic and abiotic stresses. *L. perenne* and some *Festuca* species are colonized by a group of e-endophytes consisting of the fungi from the genus *Epichloe* and *Neotyphodium*. They protect host plants from herbivores, parasites and drought. These symbionts stimulate the grasses to produce loline alkaloids including nonfluorescent perloline of *L. perenne* (Powers and Ponticello 1968; Wilkinson et al. 2000). Annual ryegrasses including *L. multiflorum* are colonized by a different group of fungi, a-endophytes represented by parasitic species of *Acremonium* (Malinowski and Belesky 2000). Their physiological role is not known and it can be only speculated that different endophytes in Italian and perennial ryegrass stimulate the synthesis of different alkaloids such as fluorescent annuloline in the former and nonfluorescent perloline in the latter. One caution to this explanation should be made that *Festuca* species produce fluorescent alkaloids although they are colonized by the same fungi as *L. perenne*. Alternatively, the very low level of loline alkaloids in *L. perenne* may suggest lower level of infection in comparison with *Festuca* or infected plants do not produce alkaloids.

Perhaps in cooler environments, to which *L. perenne* is adapted, the effect of herbivory is not so strong. Because the benefits related to alkaloid-mediated reduction of competition or herbivory are not realized, the costs of alkaloid production are limited (Faeth 2002). This explanation seems also plausible for *L. persicum*. This species is a noxious weed of oat that is known from allellopatric properties. As a weed it can take advantage from the host alkaloids not bearing any costs. A serious difficulty in all explanations of fluorescence in the genus *Lolium* is unknown physiological role of annuloline. Its structure is clearly different from perloline, it is only sparingly soluble in water (Axelrod and Belzile 1958) and presumably it does not act as an osmoregulator. However, cinnamic acid, the parent compound of annuloline can act as optical filters by blocking UV-A and B ("Cinnamic acid", 2006). Perhaps UV light penetrating soil layers could photoactivate fluorescent annuloine to create a defence response. To cite one example, it is documented that insecticidal activity of fluorescent alkaloids secreted by *Oxalis tuberosa* (oca) is linked to photoactivation. The strongest fluorescence is correlated with resistance to the larvae of *Mycrorypes* and it is observed in cultivars from the Andean highlands, which are subjected to a high incidence of UV radiation (Walker et al. 2003).

Regardless fluorescence is a species specific marker or not, it enables to distinguish between two different functional types i.e., between annual, fast growing Italian ryegrass and perennial ryegrass. And despite of variation in the fluorescence test it can be used as a determinant trait in seed certification schemes to track mechanical seed contamination. Because the majority of *L. perenne* plants do not fluoresce, the elimination of fluorescent seeds, irrespective they are *L. perenne* or *L. multiflorum* assures that none undesirable plant will be in a seed sample. This can be a pragmatic way to prevent from low turf quality. However, the more basic way to distinguish crops or even cultivars is to identify DNA fragments or genes that are responsible for differences. The three RAPD markers identified in the present work and associated with fluorescence can be beneficial to developing such DNA tests. These tests may reduce the problems caused by environmental variation and subjectivity in scoring results. A striking example of a DNA marker used in separation of different phenotypes is the SSR marker, *sat309* located only 1-2 cM away from the *rhg1* responsible for the resistance to soybean cyst nematode. Genotypic selection with this marker is 99% accurate in predicting susceptible lines (Babu et al. 2004). The other successful cases include two RAPD markers tightly linked to a gall midge resistance gene (*Gm2*) in rice (Nair et al. 1995), two RAPD markers flanking the locus *Lr19* responsible for the leaf rust resistance in wheat (Gupta et al. 2006) and several markers based on single nucleotide polymorphism of coding and adjacent noncoding regions of *Pm3* alleles conferring resistance to powdery mildew in wheat, *Triticum aestivum* and spelt, *T. spelta* (Tommasini et al. 2006). RAPD markers found in the present work offer a quick and easy way of discrimination between *L. multiflorum* and *L. perenne*. Nonetheless, they suffer some of the same problems as the seedling root fluorescence e.g., they are not valid for all cultivars. Much more accurate and flexible test can be developed provided that DNA markers linked to key morphological characteristics are identified. There are some morphological characteristics that differentiate *L. multiflorum* and *L. perenne* such as the 1st year ear emergence and recovery after winter (Chapter 3). Based on the knowledge about the chromosome locations of QTLs underlying these characters far more DNA tests can be developed.

5.5. CONCLUSIONS

1. Seedling root fluorescence can be used as a marker of kind to assess the level of annual ryegrass contamination in perennial ryegrass seed lots.
2. Genes encoding seedling root fluorescence are present in the gene pool of *L. perenne* however, with much lower frequency than in *L. multiflorum*. Presumably two complementary genes are responsible for fluorescence.
3. Fluorescence is not correlated with morphological characters typical of *L. multiflorum* although the linkage between fluorescence and QTLs can not be excluded prior to mapping studies.
4. Seedling root fluorescence is a feature of the whole genus *Lolium*. Its presence is probably related with evolutionary history and defence mechanisms.
5. DNA markers associated with fluorescence can bypass the difficulties related with fluorescence and thus, can be an alternative test for seed quality assessment.

6. DO ANY SPECIES BOUNDARIES EXIST BETWEEN *L. MULTIFLORUM* AND *L. PERENNE*: OUTCOMES FROM GENETIC MAPPING?

6.1. INTRODUCTION

Speciation is inevitable associated with the erection of reproductive isolation barriers. This process is strongly affected by the number and magnitude of genetic changes required for the evolution of isolation mechanisms. A well body of morphological and molecular data has shown extensive gene flow between two botanical species, *L. multiflorum* and *L. perenne* (Chapter 3-5) and has confirmed the lack of reproductive barriers between them. However, full reproductive barriers evolve gradually and they are seldom absolute between closely related taxa. Thus, one of the major challenges with respect to *L. multiflorum* and *L. perenne* is to understand at what point of these evolutionary processes they are. The traditional approaches to addressing formation of reproductive barriers have involved observations of their co-segregation with mapped morphological or biochemical markers. Unfortunately, such studies have necessarily been limited to model species with well-known genetic systems. The recent availability of DNA markers covering the whole genome has permitted, for the first time, to elucidate mechanisms underlying genetic differentiation during speciation. In economically important plants studies of species barriers have taken advantage of well-saturated genetic maps that provide means of assessing the number and distribution of genetic factors directly contributing to the erection of reproductive barriers (Rieseberg et al. 1995; Harushima et al. 2001). Genetic mapping studies provide an opportunity to address the fundamental question in considerations about the taxonomic status of *L. multiflorum* and *L. perenne*: does any reproductive isolation barrier exist between them?

Reproductive isolation barriers can be achieved by a variety of mechanisms at various stages in the life history of species. Prezygotic barriers prevent the formation of a zygote by ecological (or habitat), temporal, ethological, and mechanical isolation as well as by incompatibility. By contrary, postzygotic barriers prevent a zygote from developing into fertile offspring and it is observed as F_1 inviability, sterility or hybrid breakdown in which F_1 hybrids are robust and fertile, but later generation hybrids are weak and inviable. Reproductive barriers under the biological species concept are considered biological factors (postzygotic) rather than resulting from geographical or ecological separation alone (Avise 2004). At the beginning, genetic differentiation might involve changes in gene regulation mediated by relatively few control elements, negative epistatic interactions between divergent alleles (Bouck et al. 2005), re-mobilisation of transposons (Chapter 4) as well as cryptic structural rearrangements in chromosomes (Rieseberg and Carney 1998; Chetelat et al. 2000). Such changes may be so subtle that they can hardly be distinguished at the genetic diversity level.

The nature of processes that occur at the early stages of speciation can be understood by investigating the patterns of non-Mendelian inheritance of molecular markers in linkage mapping populations. The hybrid progeny may receive more alleles from one parent than expected under Mendelian rules. For example, segregation ratios in crosses between pearl millet, *Pennisetum glaucum* and *P. violaceum* can be skewed 12:1 in favour of *P. violaceum* alleles (Liu et al. 1996). In crosses between *Iris fulva* and *I. brevicaulis* significant ratio distortion has been detected in spite of the fact that F_1 hybrids are vigorous, highly fertile and undergo normal meiosis (Bouck et al. 2005). Notwithstanding these striking examples, a cautionary point related with interfering evolutionary relationships from marker distortions alone is that the tempo of changes is differing throughout the whole genome. Alleles with neutral or positive effects will likely segregate according to Mendelian rules even in crosses between relatively distant species. Despite the fact that hybrids between *Lycopersicon esculentum* (tomato) and *Solanum lycopersicoides* (wild nightshade) display incomplete chromosome pairing and high pollen sterility, the majority of loci fit Mendelian expectations in the F_2 generation. The segregation distortions, in favour of homozygotes on chromosomes 2 and 5 and heterozygotes on chromosomes 6 and 9 have eventually been revealed by mapping studies (Chetelat 2000). These studies have also documented severe recombination suppressions, sometimes along the entire length of chromosomes. Another remarkable discovery of a unique segregation distortion locus (D) in the cross between *Mimulus guttatus* and *M. nasutus* (monkeyflowers) exemplifies how genetic maps can help to assess the number and distribution of genetic factors affecting interspecific hybridization. At the most distorted marker in the D region only 1% of individuals in the F_2 mapping population were *M. nasutus* homozygotes. The magnitude of distortions diminished proportionately at increasingly distant markers (Fishman and Willis 2005). Genetic map based reappraisals have also provided support for postulated hybrid origin of *Helianthus anomalus*. Applying 197 mapped molecular markers, Rieseberg et al. (1995) were able to identify the precise linkage blocks in this species that had stemmed from the parental species i.e., *H. annuus* and *H. petiolaris*. Thus, genetic maps based on interspecific crosses provide insight into the genetics of divergence and reproductive barrier formation in incipient species.

Until recently, there are no studies of species boundaries between *L. multiflorum* and *L. perenne* at the level of details provided by a genetic mapping approach. This is partly because there have been relatively few comprehensive genetic maps for interspecific crosses (Hayward et al. 1998; Warnke et al. 2004). Two interspecific maps that have already been published suffer several limitations including the complex composition of mapping populations, relatively low resolution and high number of unallocated markers. Most mapping studies, taking at face value the economical importance of ryegrasses, tend to focus on developing molecular marker systems for *L. perenne*, which can be used for marker assisted selection. To address this, the International *Lolium* Genome Initiative (ILGI) was established to construct an enhanced genetic map of *L. perenne*. At present this map contains RFLP loci detected by heterologous probes from wheat, barley, oat and rice as well as SSR and AFLP loci (Armstead et al. 2002). It has also been aligned with maps of wheat, barley and oat, revealing substantial synteny with the genomes of Poaceae (Sim et al. 2005). Notwithstanding the substantial progress made by ILGI in *Lolium* genome mapping, this initiative has hampered further mapping studies based on various mapping populations in different *Lolium*

species. Indeed, the majority of *L. perenne* maps have been based on a single mapping population, the p150/112 that was created at the Institute of Grassland and Environmental Research (IGER) and distributed to ILGI participant laboratories for genetic analysis (Jones et al. 2002b). Two other maps published have also been developed as successors to the p150/112 (Armstead et al. 2002; Faville et al. 2004). Given the speciation perspective all these maps have serious weaknesses. First, being based only on *L. perenne* they tell nothing about evolutionary relationships between *L. multiflorum* and *L. perenne*. Second, a very complex descent of the p150/112 mapping population makes it more predisposed to investigation of breeding history in parental cultivars than species history. A doubled haploid line (DH290) derived from the Danish cultivar "Verna" was used as the female parent of the p150/112 population. The pollinator was the highly complex *L. perenne* hybrid derived from a cross between a Romanian accession and a plant from a polycross of an Italian accession and cultivar Melle (Bert et al. 1999). In summary, at least four accessions gave rise to this population that ensures high heterozygosity and marker segregation but makes difficult evolutionary considerations. Each accession represents its own breeding history with introgressions from various sources and rearrangements resulting from various breeding methodologies e.g., anther cultures.

Therefore, the objective of the present experiments was to construct an enhanced molecular marker-based genetic linkage map for studying genomic interactions and reproductive barrier formation between *L. multiflorum* and *L. perenne*. The first question was whether there are marker distortions in this interspecific cross; and, if so, what is the magnitude of distortions. Taking into account the reasonable size of the experiment, as far as it was possible, comparisons with intraspecific crosses were made. The second question was where distorted markers are located and whether they are clustered in specific chromosome regions or not.

For the first time, the mapping family was based on a F_2 population that was developed from a single F_1 plant as it is done in the majority of crops. Genetic segregation in such F_2s is the result of the meiotic recombination in a single F1 genotype that ensures that only two alleles segregate in the mapping population. In outcrossing species difficulties are encountered in the development of such populations owing to self-incompatibility and the lack of homozygous parental lines. Therefore all *Lolium* maps are constructed based on more or less complex populations originating from several heterozygous plants. In this procedure, apart from complicated segregations and difficulties in appropriate software usage, two independent maps, male and female have to be constructed and further joined to produce a sex-average map. On the other hand, even if it is time consuming, the self-pollination of *L. multiflorum* and *L. perenne* hybrids is not impossible. Both species are not obligatory outbreeders and various levels of self-pollination ranging from 20% to 80% have been observed in cultivars and ecotypes (K. Polok, unpublished data). Therefore, especially in interspecific crosses, there is no reason to use complex populations for mapping studies.

Apart from all these limitations, *Lolium* maps are predominantly based on RFLP as anchor markers. Although RFLP probes are well suited for comparative mapping, their usage encounters at least two problems. First, screening large numbers of probes across many diverse species may be troublesome. To this point sequence tagged site markers (STS) based on PCR reaction may be more practical. STS markers are generated by designing

primers from a known sequence of the target genome and they can be readily transferred between closely related species. For this reason STS markers may offer a reliable system for mapping orthologous loci and aid in the alignment of genetic linkage maps (Taylor et al. 2001). Therefore a final objective of this part of studies was to map a number of STS markers derived from *Lolium* and cereals sequences. Apart from them, a new approach was proposed that employ primers complementary to conservative bacterial sequences as for example catalase-peroxidase gene (*KatG*) or insertional elements. Such primers have proved to amplify reproducible polymorphism in many plant taxa and are useful as species specific markers (Zielinski and Polok 2005). When two species have different banding patterns revealed by bacterial specific primers, the encoding sequences can be mapped in interspecific crosses what can further inform about genomic regions responsible for species divergence. From one side, mapping of markers generated by bacterial specific primers would confirm our observations that descendants of bacterial genes do exist in plant nuclear genome, and from another it would support the utility of this approach for evolutionary studies in plants.

Second, RFLP based maps tend to focus on unique sequences and probes. When they map to more than one location they are very often discarded from analyses as artefacts. Such a strategy entails inevitable to overestimating the level of synteny between closely related species (Bennetzen 2000). Much of the differences between genomes of closely related species are attributable to repetitive sequences. The first RFLP map generated for sorghum disclosed large conserved genic regions in comparison with maize. In contrast, the interspersed regions were highly diverged (Bennetzen and Freeling 1997). This dynamism of intergenic regions results from rapid turnover among transposable elements. Linkage maps based on transposons provide insight into the pattern of genetic incompatibilities between closely related taxa including *I. fulva* and *I. brevicaulis* (Bouck et al. 2005). Unfortunately, up to date the transposon approach has been rarely used in mapping studies of cereals, and none insertional polymorphism has been map in *Lolium* yet. This is the first attempt to map insertion sites related with both DNA and retrotransposons in *L. multiflorum* and *L. perenne*.

6.2. MATERIAL AND METHODS

6.2.1. Magnitude of marker distortions in intra and interspecific crosses

Plant material

To select the most polymorphic population for mapping studies and to estimate the marker distortions at intra- and interspecific level four F_2 families were compared. The detailed description of these families, crossing experiments and plant development are given in Annex 13.1. Shortly, two populations, namely HU5 x BO2 and BR3 x NZ15 were derived from interspecific crosses. The former originated from a cross between a Hungarian ecotype of *L. perenne* and the cultivar of *L. multiflorum*, Bartissimo. The latter was derived from the cultivar of *L. multiflorum*, Bartolini and a *L. perenne* ecotype from New *Zea*land. Moreover two intraspecific populations were included in the analysis. The population coded VA7 x AS17 originated from *L. multiflorum* and was derived from a cross between an Italian ecotype, Variamo and the cultivar, Asso. The population coded KY20 x BB6 originated from

L. perenne and was derived from a cross between the cultivar Kyosato and breeding line Ba012 obtained from the Breeding Station in Bartazek, Poland. To assure that genetic segregation observed in the mapping populations was the result of the meiotic recombination in a single genotype each population was developed by selfing a single F_1 hybrid plant. Similarly, each F_1 hybrid resulted from a single cross between two individuals. This crossing design ensured the 3:1 (dominant markers) or 1:2:1 (codominant markers) segregation in F_2 populations irrespective of parental genotypes (Table 6.1).

Table 6.1. Theoretical segregation in F_2 populations depending on genotypes of parents and F_1 hybrids

N	Genotypes				Segregation in F_2	
	P_1 (female)	P_2 (male)	F_1	F_2	Dominant	Codominant
1	AA	aa	Aa	AA Aa aa	3 1	1 2 1
2	Aa	aa	Aa	AA Aa aa	3 1	1 2 1
			aa	aa	no segregation	no segregation
3	Aa	Aa	AA	AA	no segregation	no segregation
			Aa	AA Aa aa	3 1	1 2 1
			aa	aa	no segregation	no segregation

Comparison to expected ratios

To maintain a realistic experimental size only isozymes and seedling root fluorescence were used in order to compare the magnitude of marker distortions in intra- and interspecific populations. Seedling root fluorescence (SRF) was analysed on the 14th day after germination according to the methodology described in Annex 13.2. Enzyme analysis was carried out on frozen leaves harvested about four weeks after the autumn cut. Various electrophoretic techniques were employed to detect 17 different enzymes by methods described in Annex 13.3.

Agreement with the expected Mendelian segregation ratios was evaluated using the χ^2 goodness-of-fit test with the degrees of freedom equal to the number of observed classes minus 1. Observed frequencies of all isozyme loci were compared to expected genotype frequencies segregating in 1:2:1 ratio, dominant DNA markers were checked against 3:1 ratio and seedling root fluorescence both against 3:1 and 9:7. Each locus was tested individually.

6.2.2. Genetic map construction

Selection of interspecific mapping population and DNA marker analysis

To insight into the level of genetic incompatibilities between *L. multiflorum* and *L. perenne* a genetic map was constructed based on an interspecific population. At the beginning two interspecific crosses were compared, namely HU5 x BO2 and BR3 x NZ15 by means of DNA markers. Intraspecific crosses were also included in this preliminary analysis. The screening panel consisted of parents, F_1 hybrids and six F_2 individuals. In total 60 RAPD and 12 ISJ primers were tested. Based on the results of these analyses, the interspecific F_2 family coded BR3 x NZ17 was selected for genetic mapping because it was the most polymorphic population. Genetic similarity between parents of this combination was as low as 0.588 comparing with 0.754 between HU5 and BO2.

The mapping population consisted of 269 genotypes. DNA was isolated from frozen leaves of F_2 individuals using modified CTAB method (Annex 13.4). To obtain a high resolution map suitable for evolutionary studies and further QTL analysis, apart from previously selected RAPD and ISJ markers, a wealth of different DNA markers was added including those generated by arbitrary chosen primers (AFLP), markers based on simple sequence repeats (SSR) and transposon sequences (SSAP). In total seven pairs designed for SSR loci, 34 AFLP primer combinations (*Mse*I and *EcoR*I specific), and six SSAP combinations including three combinations specific to *Tpo1* DNA transposon and three combinations specific to *Lolcopia2* retrotransposon were tested. In all SSAP combinations, either primer specific to the *Mse*I restriction site or *Pst*I restriction site was used as a second one. SSR primers were taken from Jones et al. (2001) and named accordingly as A02, A07, A10, A11, C11, E10, and H06. They were chosen because they revealed high level of polymorphism in different accessions of *L. perenne* and cross-species amplification was detected in a number of grasses. To increase the possibility of alignment with the Poaceae maps 13 sequence tagged site markers (STS) were generated by using primers complementary to known sequences from different *Lolium* species and cereals. A number of STS markers were used that have previously shown amplified polymorphism in *L. multiflorum* and *L. perenne* ecotypes (Zielinski et al. 1997). They were derived from a complete sequence of *L. perenne* pollen allergen genes, Lol p I (LOLPISO5A); 3' end of Lol p I (LOLPISO1A), Lol p Ib sequence (LOLLOPIB) and *L. temulentum* nuclear gene encoding chlorophyll binding protein type II (LTLHAB). Another set of STS markers was taken from Taylor et al. (2001) based on amplification and restriction site polymorphism. They were derived from L-asparaginase (ASNL), thioredoxin (Trx) and glutamine synthetase (Gln2) of *L. perenne*, L-asparaginase (ASN), asparagine synthetase (AS1) and HS1 protein of *H. vulgare*, a RFLP probe, BCD450 from *H. vulgare* and RFLP probes, CDO504 and CDO1508 from *A. sativa*.

Four types of markers derived from *M. tuberculosis* sequences that were effective in differentiation of closely related species at different taxonomic levels (Zielinski and Polok 2005) were used to map some of regions responsible for species divergences. Pairs of primers were designed on hot fragments of *rpo* and *pol* genes and a pair of outer primers was based on terminal repeats of insertion element IS6110. Twelve pairs of primers (katG1-katG12) were complementary to catalase-peroxidase gene (*KatG*). These types of markers

were further assigned as B-SAP (Bacteria Specific Amplification Polymorphism). Sequences of all primers used in the mapping studies are given in Annex 13.5 whereas amplification and electrophoresis conditions for all marker types are described in Annexes 13.8-13.14.

Linkage analysis and software used

Linkage between enzymatic loci was analysed in four F_2 populations whereas the linkage map was constructed for the BR3 x NZ15 interspecific population. To determine if two loci are linked, the logarithm to the base of ten of the likehood odds ratio statistics was used (LOD). This statistics informs about the conditional probability of an odd number of cross-overs between the markers. The LOD score was calculated for various recombination values (Θ). The (Θ)value, which gives the highest LOD score, was taken as the best estimate of the recombination fraction. A LOD score of 3 or greater for the recombination fraction between two loci lower than 0.49 confirmed the linkage. A LOD score of lower values excluded linkage.

The programme MAPL98 (Ukai 2004), originally developed for rice genome mapping, was used first. It grouped markers by connecting a marker A with another marker B which had the smallest recombination value with the marker A. Starting with an arbitrary chosen marker the connection was repeated until no partner was found and a chain of markers was constructed. A marker at the end of the chain was judged as having no partner with a recombination value lower than a threshold value. The threshold value was determined so that probability of marker connection by error was 0.05. Multidimensional scaling allowing finding the shortest way between markers was used for final marker ordering. Then map distances were calculated using Kosambi's mapping function, which allowed for modest interference (Kosambi 1944). At the second stage of analysis, the programme Carthagene (Schiex et al. 2005) was employed. This software was comparable with the more widely used MAPMAKER but it worked in WINDOWS XP environment and used WINDOWS interface. Additionally it allowed comparing different maps. The map was drawn with help of MapChart (Voorrips 2002) and transformed into CorelDraw 12 (2004) format in order to prepare it for printing in high resolution.

Due to huge amount of data, the map was built successively by separate mapping of each marker, and combining the maps into a single linkage map using MAPL98. Final mapping was done for each linkage group separately. Markers were coded as 1 for a female and 2 for a male type. Additionally, for codominant markers a heterozygous type was recognised as 3. For some dominant markers segregating in F_2, parental phenotypes were identical i.e., both had a band. In these cases F_1 and parental plants must have been heterozygotes for null alleles. This was owing to the fact that *L. multiflorum* and *L. perenne* are allogamous species and therefore parental plants were not completely homozygous lines. Consequently, linkage phase was unknown in a certain part of data. In these cases the presence of a band was arbitrary coded as 1 and the lack as 2. Then counterpart data in which "1" and "2" were mutually converted were made and linkage analysis was done on the original and counterpart data. The underlying assumption was that the markers with incorrect phase assignments would not be linked to any of the markers where the correct phases were known. For a given marker category, first all linked markers were checked altogether and then separately. This set of data was selected that produced fewer groups, shorter map and fewer solitary mark-

ers. Then the procedure was repeated for all solitary markers. Conversion was also repeated each time when a new marker category was introduced onto the map. To produce a suitable robust genetic map the final order of markers was transformed into the Carthagene format using Excel (2003) transposition. Linkage groups were again determined using 2-points estimation and a minimum LOD ratio of 6.0 and a maximum recombination rate of 0.2. Optimal order was estimated using the annealing command and verified using validation methods as implemented in Carthagene. Once the map was constructed, marker coverage was compared with that of existing maps.

6.2.3. Numbering of linkage groups

The linkage groups were numbered in accordance with the corresponding linkage groups of the first molecular marker based map of perennial ryegrass published by Hayward et al. (1998) owing to the number of enzymatic loci. Consequently, *Est4* and *Aat2* determined linkage group 1 (LG1), *Hk*1 - LG2, *Gtdh*2 - LG5. Alignment of LG3, LG4, LG6 and LG7 was problematic due to limited number of common markers detecting a single locus and therefore these groups were numbered arbitrary, yet taking into account the overall length of a given linkage group. This numerical order was chosen because it was partially in agreement with the chromosome numbers as allocated by Lewis et al. (1980) with primary trisomics. Alignment with the ILGI *L. perenne* map was troublesome due to inconsistency in linkage group numbering by different laboratories and different marker naming. For example Bert et al. (1999) used alphabetical order for their AFLP map constructed in the p150/112 population. The first SSR map of this population constructed by Jones et al. (2002a) employed numerical order. Surprisingly, the same authors using also the p150/112 population numbered AFLP linkage groups on the basis of conserved synteny with Triticeae (Jones et al. 2002b). Consequently, the numerical order in their AFLP map was in agreement neither with the previously published SSR map nor with the AFLP map of Bert et al. (1999). What is more, Jones et al. (2002b) described that *Mse*I/*EcoR*I primer combinations were used in AFLP analyses, whereas names of AFLP loci on their map suggesting that they used *Tru*9I/*EcoR*I combinations. Similar problem with the alignment of the ILGI linkage map was experienced by Muylle et al. (2005) due to the detection of multiple loci by the respective SSR or RFLP probes.

6.2.4. Marker naming on linkage map

Due to large number of markers on the linkage map, and to keep the map readable, some abbreviations had to be introduced (Table 6.2). In general, enzymatic loci were indicated according to the names of their enzymes as indicated in Annex 13.3. DNA loci were indicated by marker type and product number counting from the fastest band. AFLP loci were named according to restriction sites flanked by adapters (*Mse*I, *EcoR*I) and three-base extensions of selective primers. Numerical nomenclature as it is on the ILGI map and some others was avoided to improve readability without necessity to look at the standard list for AFLP primer nomenclature. The same idea was employed for transposon-based primers.

Table 6.2. Marker codes as they appeared on the genetic map of *L. multiflorum* and *L. perenne*

Marker type	Abbreviation format	Example
Seedling root fluorescence	SRF	SRF
Isozymes	*Xxx1* (locus name and number)	*Est1*, *Sod1*
RAPD	OPAXX-Y, OPBXX-Y, OPDXX-Y	OPA08-14, OPB19-13, OPD17-1
ISJ	ISJXX-Y	ISJ8-6, ISJ12-7
SSR	AXX-Y, CXX-Y, HXX-Y	A02-1, C11-2, H06-1
AFLP	MxxxEyyyZ (M – *Mse*I primer, E – *EcoR*I primer)	MctgEaac34
SSAP-*Tpo1*	TpPatY, TpMacaY (P – *Pst*I primer, M – *Mse*I primer)	TpPat40, TpMaca32
SSAP-*Lolcopia2*	Lc2PatY, Lc2MccY (P – *Pst*I primer, M – *Mse*I primer)	Lc2Pat6, TLc2Mcc11
B-SAP – IS6110	ISXX	IS1
B-SAP - katG	katGXX-Y	katG2-14, katG3-7

6.3. RESULTS

6.3.1. Polymorphism between parents of intra-and interspecific crosses

Whether parents originated from the same or different species did not influence the level of polymorphism that was rather correlated with breeding history of the crossed individuals. For the 17 enzymes analysed, a total of 34 loci were identified in all parental plants. Among them 19 loci (55%) were polymorphic between parents of all crosses (Table 6.3). At the rest of loci but *Per2* the same alleles were observed in all analysed parental plants. Polymorphism was observed as changes in mobility for the majority of loci. The only exception was the null allele at *Est1* in HU5. The number of alleles did not differ much between parents of interspecific cross, HU5 x BO2 (18%) and both intraspecific crosses (12%). Likewise, DNA differences were comparable at the intra- and interspecific level. The genetic identity estimated from RAPD and ISJ markers was only slightly lower for the combination HU5 x BO2 (I=0.754) than for *L. multiflorum* (I=0.870) and *L. perenne* (I=0.842) combinations. The only exception was the interspecific cross, BR3 x NZ15, whose parents were highly diverse having different enzymatic alleles at 16 enzymatic loci (37%) and DNA based genetic similarity as low as I=0.588. Such great differences between BR3 and NZ15 parents were also confirmed by analyses of other DNA markers including SSR, AFLP, SSAP and bacteria derived-markers, B-SAP (Figure 6.1). Of the 147 different kinds of primers or primer combinations 43 (29%) generated various level of polymorphism. They amplified 1018 fragments in total, and among them 261 were polymorphic (25%) between parents. Another 223 fragments were not polymorphic between parents; however parental lines should have been heterozygous for them because these fragments segregated in the F_2 population. Because of high polymorphism between parents this population was further used for genetic mapping studies.

With regard to enzymatic loci fast alleles were equally distributed between parents of interspecific crosses (Table 6.4A-B) but they were associated in one of parents in intraspecific crosses (Table 6.4C-D). Heterozygotes usually had two bands corresponding to parental bands suggesting that active enzymes are monomers. However, it should be noticed that

Table 6.3. Mobility of enzymatic alleles identified in parents and F_1 hybrids of *L. multiflorum* and *L. perenne*

Enzyme	Locus	Cross combination											
		BR3 x NZ15			HU5 x BO2			VA7 x AS17			KY20 x BB6		
		BR3	NZ15	F_1	HU5	BO2	F_1	VA7	AS17	F_1	KY20	BB6	F_1
ACP	*Acp3*	28	28	28	28	28	28	28	28	28	28	28	28
ACOH	*Acoh1*	32	28	32/28	35	32	35/32	32	32	32	32	32	32
ADH	*Adh1*	30	26	30/26	26	26	26	30	30	30	26	30	30/26
AAT	*Aat1*	43	43	43	43	43	43	43	43	43	43	43	43
	Aat2	32	36	36/32	36	32	36/32	36	32	36/32	32	36	36/32
	Aat3	07	07	07	07	07	07	07	07	07	07	07	07
CAP	*Cap1*	47	47	47	47	47	47	47	47	47	47	47	47
	Cap2	42	39	42/39	42	42	42	42	42	42	42	42	42
EST	*Est1*	12	12	12	null	15	15	12	12	12	12	12	12
	Est2	35	35	35	35	35	35	40	35	40/35	35	35	35
	Est3	28	28	28	22	22	22	22	28	28/22	22	22	22
	Est4	45	49	49/45	45	49	49/45	49	49	49	45	49	49/45
EST-flu	*Est-flu1*	13	13	13	13	13	13	13	13	13	13	13	13
	Est-flu2	34	34	34	34	34	34	34	34	34	34	34	34
	Est-flu3	47	45	47/45	47	47	47	47	47	47	47	47	47
	Est-flu4	51	51	51	51	51	51	51	51	51	51	51	51
GPI	*Gpi1*	30	30	30	30	30	30	30	30	30	30	30	30
GTDH	*Gtdh1*	22	22	22	22	22	22	22	22	22	22	22	22
	Gtdh2	17	14	17/14	14	14	14	14	14	14	14	14	14
HK	*Hk1*	42	35	42/35	35	35	35	35	35	35	35	35	35
IDH	*Idh1*	27	31	31/29/27	27	31	31/29/27	27	27	27	31	31	31
MDH	*Mdh1*	42	42	42	42	42	42	42	42	42	42	42	42
	Mdh2	32	34	34/32	34	34	34	34	34	34	32	34	34/32
MDHP	*Mdhp1*	33	33	33	33	33	33	33	33	33	33	33	33
	Mdhp2	25	21	25/23/21	25	25	25	25	21	25/23/21	25	25	25
PER	*Per1*	35	45	45/35	35	35	35	35	35	35	35	35	35
	Per2	32	32	32	32	32	32	26	26	26	32	32	32
	Per3 (K)	16	10	16/10	16	16	16	16	16	16	16	16	16
PGM	*Pgm1*	47	47	47	47	47	47	47	47	47	47	47	47
PGDH	*Pgdh1*	30	34	34/32/30	30	30	30	30	30	30	34	34	34
SKDH	*Skdh1*	27	30	30/27	30	22	30/22	27	27	27	30	30	30
	Skdh2	18	18	18	18	18	18	18	18	18	18	18	18
SOD	*Sod1*	60	54	60/54	54	54	54	54	54	54	54	54	54
	Sod2	34	34	34	34	34	34	34	34	34	34	34	34
Loci polymorphic between parents		**N°**	16		**N°**	6		**N°**	4		**N°**	4	
		%	37		**%**	18		**%**	12		**%**	12	

Shadow boxes indicate loci polymorphic between parents

hybrid bands of multimeric enzymes are very often not visible on starch gels. More than two bands in F_1 hybrids were observed for IDH, MDHP and PGDH suggesting that these enzymes may be active as dimers or multimers. Distribution of DNA markers was analysed only for the mapping family, BR3 x NZ15. Parents were heterozygous for about half of DNA loci (46%), thus having identical phenotypes (Table 6.5). The other half of DNA markers were equally distributed among parents and 26% of amplified bands originated from the *L. multiflorum* female parent, while another 28% originated from the *L. perenne* male parent. Distribution of DNA markers depended in a certain degree on a marker type. Hence, the most of katG markers were provided by *L. perenne* whereas the majority of transposon-based markers, both DNA and retrotransposons, originated from *L. multiflorum*.

When different markers were compared with regard to the level of polymorphism between the most diverse parents, BR3 and NZ15, it was obvious that ISJ markers with 67%

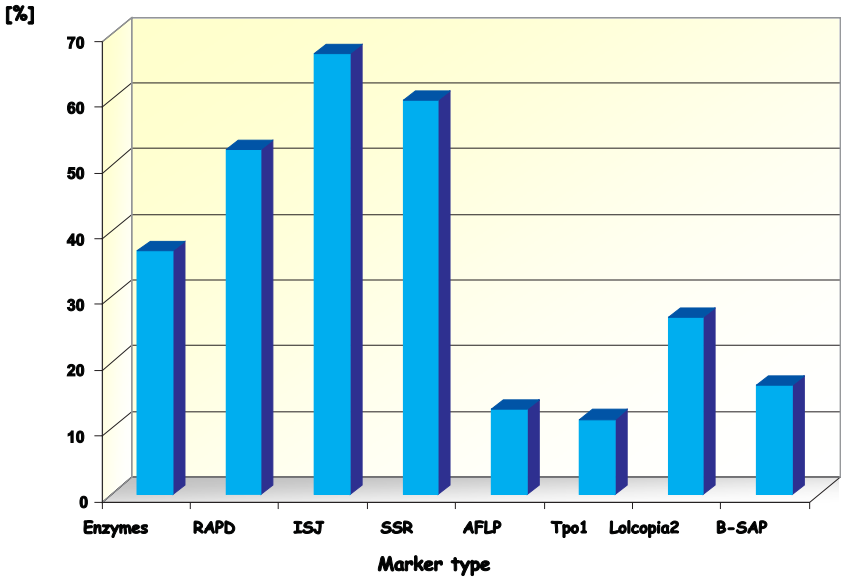


Figure 6.1. Percent of polymorphic markers between parents of the interspecific cross, BR3×NZ15

of polymorphic loci were the most effective, followed by RAPDs, and enzymes (Figure 6.1). High efficiency was also noted for SSR markers although these results could be somehow biased because only primers that had revealed high polymorphism in the studies of Jones et al. (2001) were chosen for the present analyses. Out of seven pairs of SSR primers tested, five (71%) detected polymorphic loci. Two primers produced single-locus amplification patterns, and three others detected duplicated loci (Table 6.6). In total six polymorphic loci were observed. Dominant mode of inheritance with null alleles was characteristic of SSR loci with $(CA)_n$ repeated motifs, whereas codominance was observed for loci with the other motifs. Single band alleles were observed for A02, A11, and C11 (Figure 6.2). At two SSR loci A10 and H06 alleles with a different number of bands were observed. At the former an allele, *A10-1a* had two bands whereas another had three bands (*A10-1b*). At the H06 SSR locus two- and single band alleles were found. Heterozygotes at these two loci were expected to have five (A10) or three (H06) bands however, the middle bands were very often faint or even not visible.

The most dramatic findings involved total uselessness of 13 STS markers either derived from *L. perenne* or from cereals, *H. vulgare*, and *A. sativa*. Although all primer pairs were selected on the basis of earlier studies (Taylor et al. 2001), in which they amplified a single product, in the present work optimised PCR conditions resulted in the amplification of a single product of the expected size only for 50% of sequences. Expectedly, the majority of such sequences were derived from *L. perenne* (LOLPISO5A, LOLPISO5B, ASNL, Trx) but also from *H. vulgare* (AS1) and *A. sativa* (CDO1508). The other seven primer sets could not be optimised to amplify a single band however, two strong reproducible bands were obtained in a case of four primer sets derived from *L. perenne* (LOLLOPIB, Gln2), *H. vulgare* (BCD450) and *A. sativa* (CDO504) genes. Difficulties were encountered using primers developed from

Table 6.4. Enzymatic multilocus genotypes of parents of *L. multiflorum* and *L. perenne* F_2 populations

A. BR3 x NZ15 (interspecific cross)

Parent	Polymorphic alleles between parents															
	Acoh1	*Adh1*	*Aat2*	*Cap2*	*Est4*	*Est-flu3*	*Gtdh2*	*Hk1*	*Idh1*	*Mdh2*	*Mdhp2*	*Per1*	*Per3*	*Pgdh1*	*Skdh1*	*Sod1*
BR3																
NZ15																

Fast alleles are indicated by shadow boxes

B. HU5 x BO2 (interspecific cross)

Parent	Polymorphic alleles between parents					
	Acoh1	*Aat2*	*Est1*	*Est4*	*Idh1*	*Skdh1*
HU5						
BO2						

Fast alleles are indicated by shadow boxes

C. VA7 x AS17 (intraspecific cross, *L. multiflorum*)

Parent	Polymorphic alleles between parents			
	Aat2	*Est2*	*Est3*	*Mdhp2*
VA7				
AS17				

Fast alleles are indicated by shadow boxes

D. KY20 x BB6 (intraspecific cross, *L. perenne*)

Parent	Polymorphic alleles between parents			
	Adh1	*Aat2*	*Est4*	*Mdh2*
KY20				
BB6				

Fast alleles are indicated by shadow boxes

L. temulentum gene, LTLHAB, and two *H. vulgare* genes, ASN and HS1. They all amplified several bands. Nevertheless, these multiple products were highly reproducible in parents and F_2 individuals used for screening.

Unfortunately, all STS markers were monomorphic in both parents and F_2 progeny and thus they were not useful in mapping studies. This was even though a wealth of methods was applied to disclose polymorphism. Amplification polymorphism was analysed for those primer sets that gave multiple but stable products. PCR products for single-band STS markers were analysed for restriction site polymorphism. In a case of two-band STS markers, bands were eluted from gels, re-amplified and then PCR products were subjected to restriction analyses.

In comparison with STS markers derived from Poaceae, primer sets based on *M. tuberculosis* genes proved to be quite effective in disclosing polymorphism between parents

Table 6.5. Distribution of DNA markers among parents of the interspecific F_2 mapping population, BR3×NZ15

Parameter		Method								
						SSAP		B-SAP		
		RAPD	ISJ	SSR	AFLP	*Tpo1*	*Lol-copia2*	IS6110	*katG*	Total
Markers segregating in F_2	**N°**	**132**	**80**	**6**	**137**	**57**	**39**	**6**	**27**	**484**
Markers present in the female parent (BR3)	**N°**	34	30	1	25	10	19	3	4	126
	%	25.8	37.5	16.7	18.2	17.7	48.7	50.0	14.8	26.0
Markers present in the male parent (NZ15)	**N°**	52	35	2	24	3	2	2	15	135
	%	39.4	43.8	33.3	17.5	5.3	5.1	33.3	55.6	27.9
Markers present in both parents	**N°**	46	15	3	88	44	18	1	8	223
	%	34.8	18.7	50.0	64.2	77.2	46.2	16.7	29.6	46.1

Table 6.6. SSR markers used for genetic map construction

Primer pair	Repeat motif	Repeat class	N° of loci	N° of polymorphic loci	Expected size [bp]	Observed size of alleles at each locus	Inheritance
A02	$(CA)_{27}$	Perfect	2	2	131	*A02-1*: 132*, *A02-2*: 142	Dominant (3:1) Dominant (3:1)
A10	$(CTT)_{20}$	Imperfect	1	1	152	*A10-1a*: 154, 164 *A10-1b*: 160, 185, 195	Codominant (1:2:1)
A11	$(CA)_{21}$	Imperfect	2	1	205	*A11-1*: 130, *A11-2*: 150	Homozygous Dominant (3:1)
C11	$(CA)_4TA(CA)_4$	Imperfect	2	1	198	*C11-1*: 137, *C11-2a*: 187 *C11-2b*: 197	Homozygous Codominant (1:2:1)
H06	$(CA)_9$	Perfect	1	1	150	*H06-1a*: 155 *H06-1b*: 160, 170	Codominant (1:2:1)

*Numbers after hyphen mean different SSR loci recognized by a particular primer pairs, letters mean different alleles at the same locus

of the mapping population (Figure 6.1). Among 15 primer sets derived from four bacterial sequences, five sets amplified different bands in BR3 and NZ15 that further segregated in the F_2 population (Figure 6.3). Polymorphism was generated by katG2, katG3, katG5 and katG8 primers as well as by primers complementary to IS6110. Monomorphic products were amplified by primer sets developed from *rpo* and *pol* genes. In total 145 bands were identified of which 24 bands (17%) were polymorphic between parents and further nine were present in both parents but segregated in F_2. Nearly all identified katG and IS markers segregated in 3:1 ratio in the F_2 population and the presence of a band was dominant over its lack.

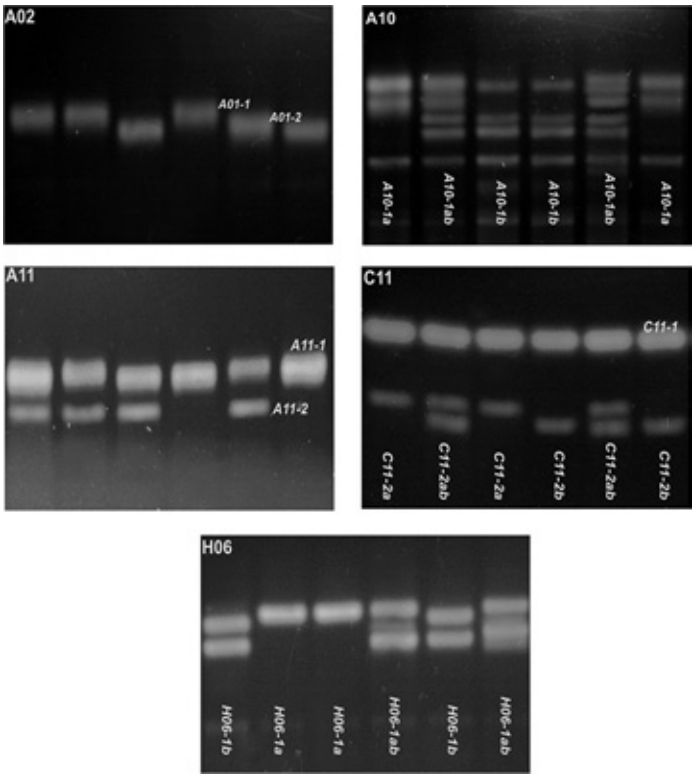


Figure 6.2. Segregation of SSR markers in the F_2 population from the cross between *L. multiflorum* and *L. perenne*, BR3×NZ15

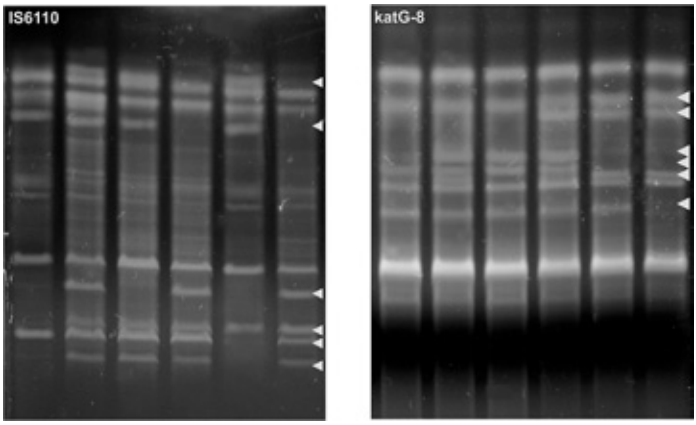


Figure 6.3. Segregation of B–SAP markers in the F_2 population from the cross between *L. multiflorum* and *L. perenne*, BR3×NZ15
IS6110 – primers complementary to *M. tuberculosis* IS6110 insertion element; katG – primers complementary to *M. tuberculosis KatG* gene encoding catalase–peroxidase

6.3.2. Genetic map of *L. multiflorum* and *L. perenne*

Genetic map was constructed based on the most polymorphic interspecific population i.e., the F_2 population derived from the cross between *L. multiflorum* cultivar Bartolini, BR3 and *L. perenne* ecotype from New *Zea*land, NZ15. The final linkage map covered 1014 cM and consisted from 502 loci including 484 DNA markers, 16 enzymatic loci and two different loci responsible for seedling root fluorescence, SRF (Table 6.7). Among DNA markers (Figure 6.4), RAPD and AFLP markers were the most abundant (132 and 137, respectively), following by transposon-based markers, SSAP (96) and Intron Splice Junction markers, ISJ (80). It was also possible to map markers amplified by primers complementary to *M. tuberculosis* insertion element IS6110 (IS markers) and *KatG* gene encoding bacterial catalase-peroxidase (katG markers).

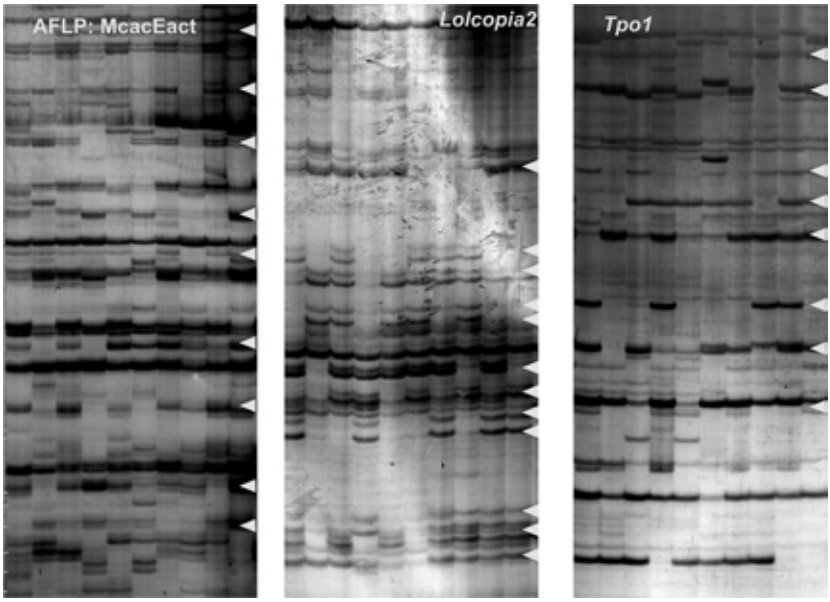


Figure 6.4. Segregation of AFLP and SSAP markers in the F_2 population from the cross between *L. multiflorum* and *L. perenne*, BR3×NZ15

All mapped markers gave rise to seven linkage groups (Figure 6.5A-G) presumably corresponding to the seven chromosomes of *Lolium*. These groups were numbered using the numerical order from LG1 to LG7, which was as much as possible in agreement with the first genetic map published by Hayward et al. (1998). The linkage groups covered from 92.7 to 214.2 cM and consisted from 46 to 91 markers (Table 6.7). The LG1 of 214.2 cM in length with 86 markers mapped was the longest among all groups (Figure 6.5A). It corresponded to the first linkage group of Hayward et al. (1998) owing to common enzymatic loci, *Est4*, *Aat2*, *Sod1* and *Pgdh1*. The presence of the majority of markers amplified by primers complementary to *M. tuberculosis* insertion element IS6110 and as many as five enzymatic loci were unusual features of this group. The LG2 (Figure 6.5B), the second group with regard to the length (183.7 cM) but the first with regard to the number of markers mapped also correspond-

ed to LG2 of the Hayward et al. (1998) map due to the presence of hexokinase locus (*Hk1*). The LG4 occupied the third place when the length of the map was taken into account (164.1 cM) but it contained comparable number of markers to LG1 and LG2 (Figure 6.5D). Similarly to LG1, five enzymatic loci were found within this group. The groups LG3 and LG5 had similar length of about 120 cM (Figure 6.4C; E) whereas LG6 was a little shorter (Figure 6.5F). The LG5, having *Gtdh2* locus corresponded to the fifth linkage group of Hayward et al. (1998). The LG3 can be characterized by a relatively low number of AFLP markers while the typical feature of LG6 was the greatest number of katG markers mapped i.e., markers amplified by primers complementary to *M. tuberculosis KatG* gene. The alignment of LG3, LG4 and LG6 with the Hayward et al. (1998) linkage groups was problematic, hence they were numbered arbitrary. The last linkage group, LG7 was the shortest (92.7 cM) with the fewest markers mapped (Figure 6.5G). Presumably, it corresponded to the LG7 of the Hayward et al. (1998) map. When compared with the other linkage groups, it consisted from the fewest number of transposon-based markers.

Table 6.7. Distribution of markers among seven linkage groups of *L. multiflorum* and *L. perenne*

Parameter	Linkage group							
	LG1	LG2	LG3	LG4	LG5	LG6	LG7	Total
Map length [cM]	**214.2**	**183.7**	**122.3**	**164.1**	**125.2**	**112.0**	**92.7**	**1 014.2**
Average distance [cM]	**2.5^{a}**	**2.0^{bc}**	**2.4^{ab}**	**1.9^{bc}**	**1.8^{c}**	**1.6^{c}**	**2.1^{abc}**	**2.0**
Total n° of markers mapped	**86**	**91**	**52**	**86**	**72**	**69**	**46**	**502**
Marker type								
SRF	2	0	0	0	0	0	0	**2**
Enzymes	5	3	0	5	1	1	1	**16**
RAPD	19	27	13	27	12	23	11	**132**
ISJ	12	13	14	11	12	6	12	**80**
SSR	2	1	0	0	2	1	0	**6**
AFLP	30	27	11	21	19	14	15	**137**
SSAP – *Tpo1*	4	14	3	11	15	7	3	**57**
SSAP – *Lolcopia2*	7	4	7	6	5	9	1	**39**
B-SAP – IS6110	4	1	0	0	1	0	0	**6**
B-SAP - katG	1	1	4	5	5	8	3	**27**

[abc]Different letters mean significant differences between mean distance on each LG at P=0.05 for LSD test

The application of several marker categories resulted in the construction of the high resolution linkage map of *L. multiflorum* and *L. perenne* with the average distance between adjacent markers of 2.0 cM (Table 6.7). The average distance in each linkage group was more or less alike, and ranged around the overall mean. However, a slightly higher distance was observed on LG1 and LG3 (2.5 cM) while the lowest one was noted for LG6 (1.6 cM). Genetic distances between consecutive pairs of markers most frequently were within 1-2 cM, less often within 2-3 cM and 3-4 cM, rarely, the distance was bigger than 4 cM (Figure 6.6). The minimum distance was observed for 33 markers that were completely linked (0 cM). The maximum distance of 10.4 cM was found between ISJ12-1 marker and *Lolcopia2* specific marker, Lc2Pat18 on LG3 (Figure 6.5C).

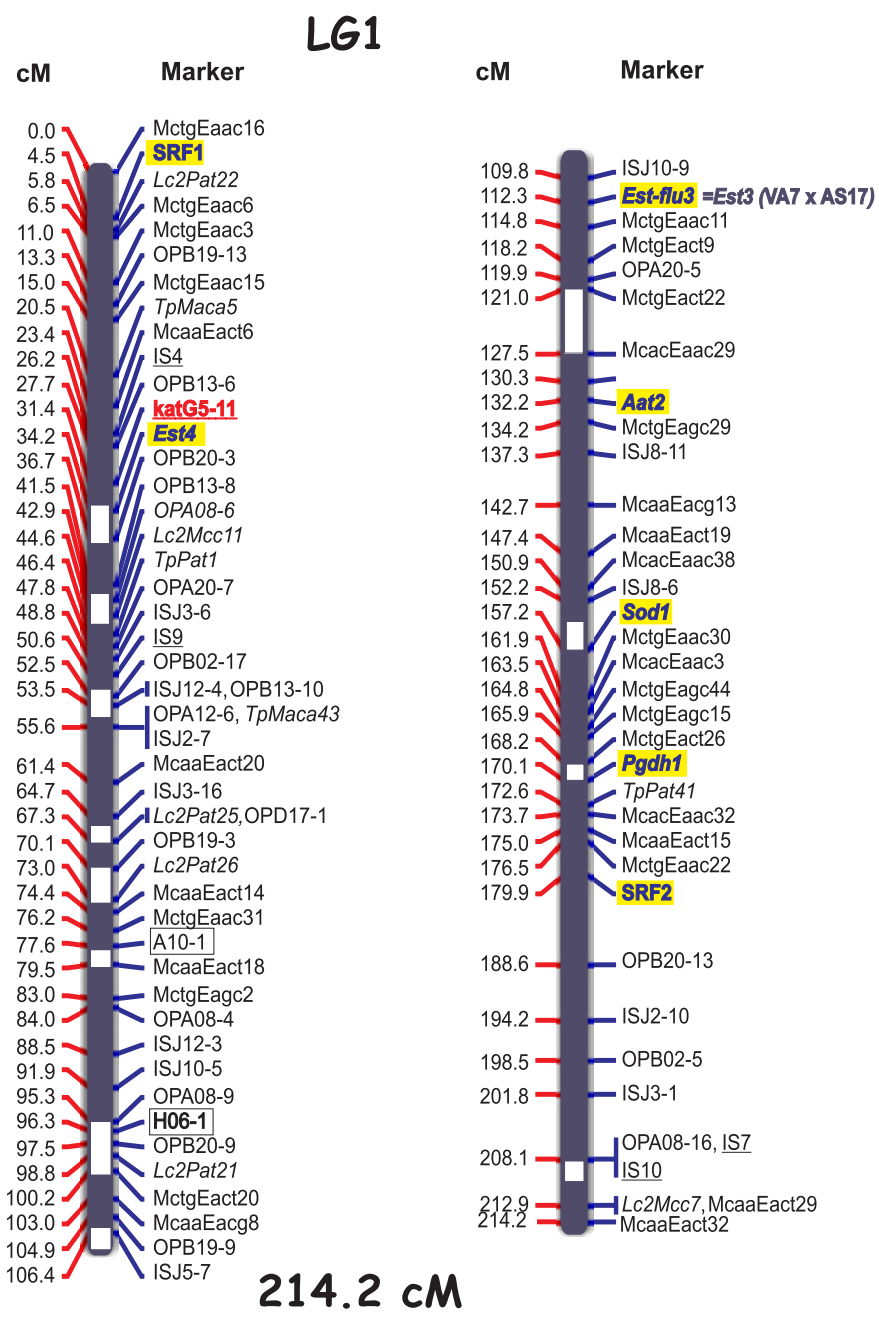


Figure 6.5A. Genetic linkage map of *L. multiflorum* and *L. perenne* based on 502 morphological, enzyme and DNA markers — linkage group LG1

- Shadow boxes, bold, italic – enzymatic loci and seedling root fluorescence (SRF), bracket – SSR loci, italic – transposon loci (Tp – *Tpo1* specific, Lc2 – *Lolcopia2* specific), bold, underlined – B-SAP loci,
- White boxes on chromosomes – regions with disturbed segregation

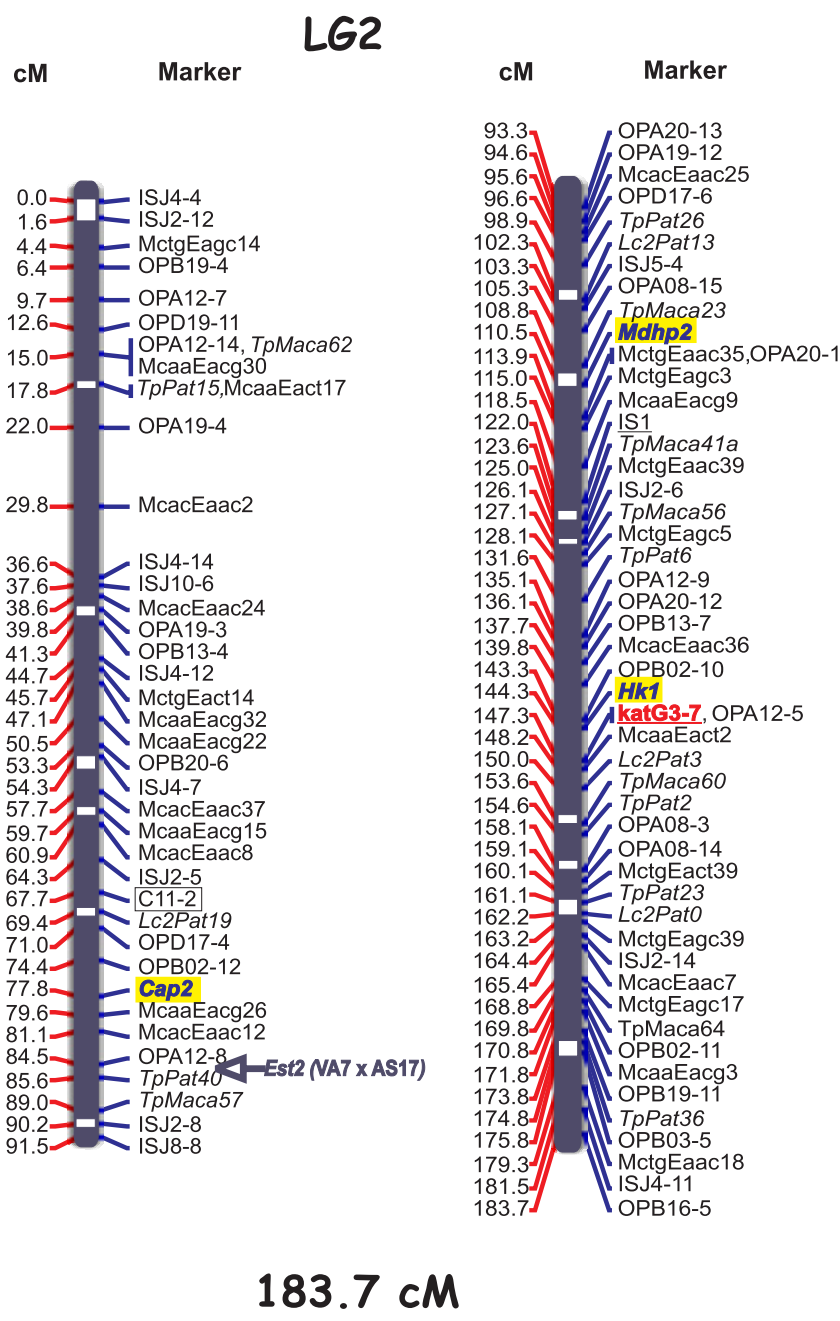


Figure 6.5B. Genetic linkage map of *L. multiflorum* and *L. perenne* based on 502 morphological, enzyme and DNA markers – linkage group LG2

- Shadow boxes, bold, italic – enzymatic loci, bracket – SSR loci, italic – transposon loci (Tp – *Tpo1* specific, Lc2 – *Lolcopia2* specific), bold, underlined – B-SAP loci,
- White boxes on chromosomes – regions with disturbed segregation

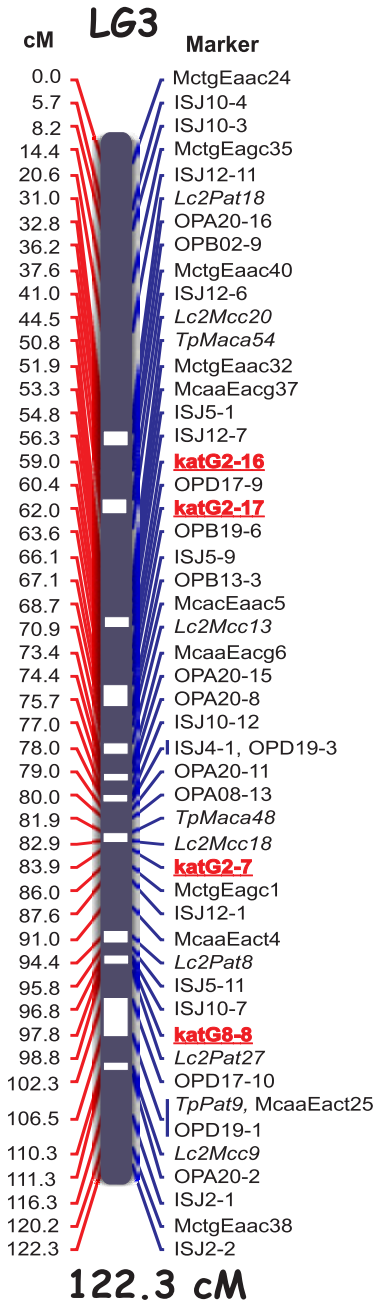


Figure 6.5C. Genetic linkage map of *L. multiflorum* and *L. perenne* based on 502 morphological, enzyme and DNA markers – linkage group LG3

- Shadow boxes, bold, italic – enzymatic loci, bracket – SSR loci, italic – transposon loci (Tp – *Tpo1* specific, Lc2 – *Lolcopia2* specific), bold, underlined – B-SAP loci,
- White boxes on chromosomes – regions with disturbed segregation

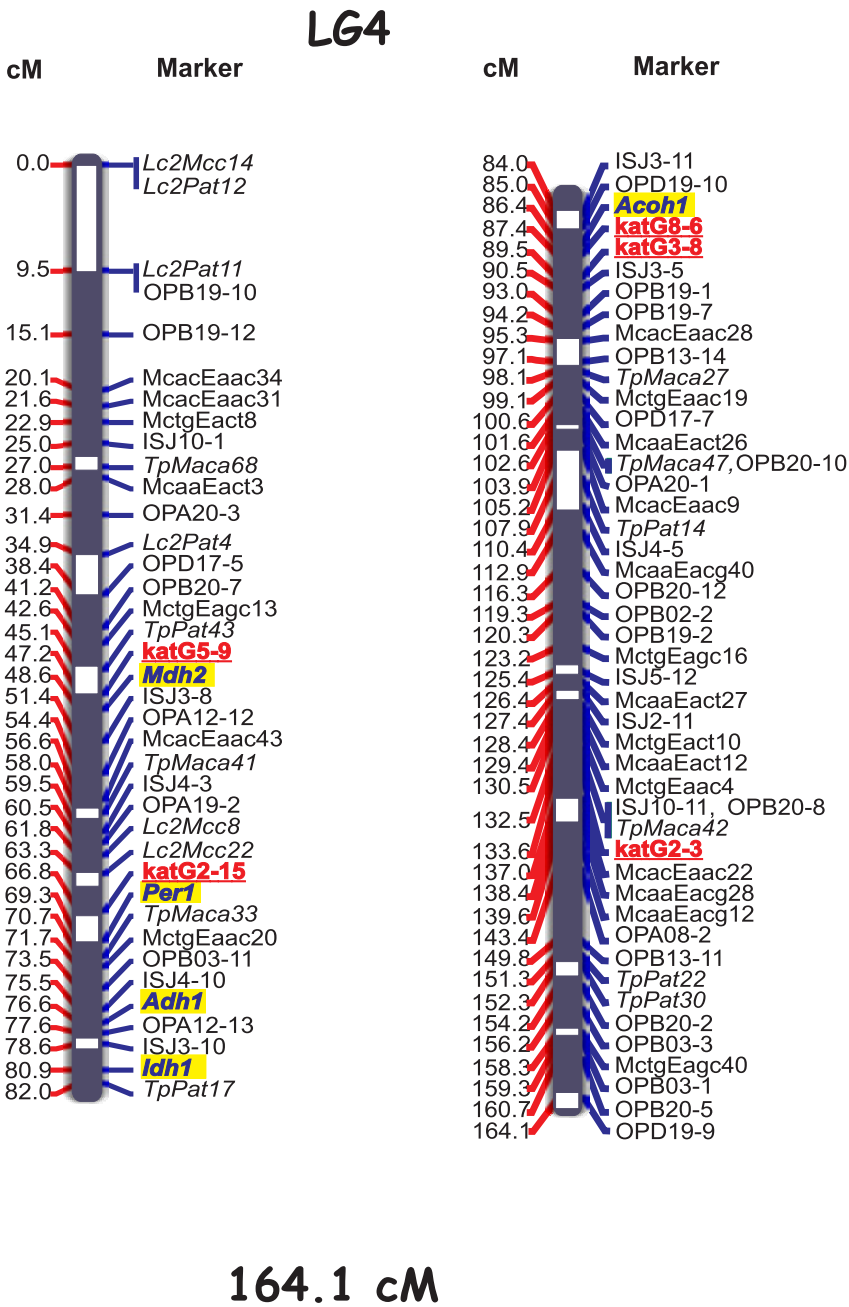


Figure 6.5D. Genetic linkage map of *L. multiflorum* and *L. perenne* based on 502 morphological, enzyme and DNA markers – linkage group LG4

- Shadow boxes, bold, italic – enzymatic loci, bracket – SSR loci, italic – transposon loci (Tp – *Tpo1* specific, Lc2 – *Lolcopia2* specific), bold, underlined – B-SAP loci,
- White boxes on chromosomes – regions with disturbed segregation

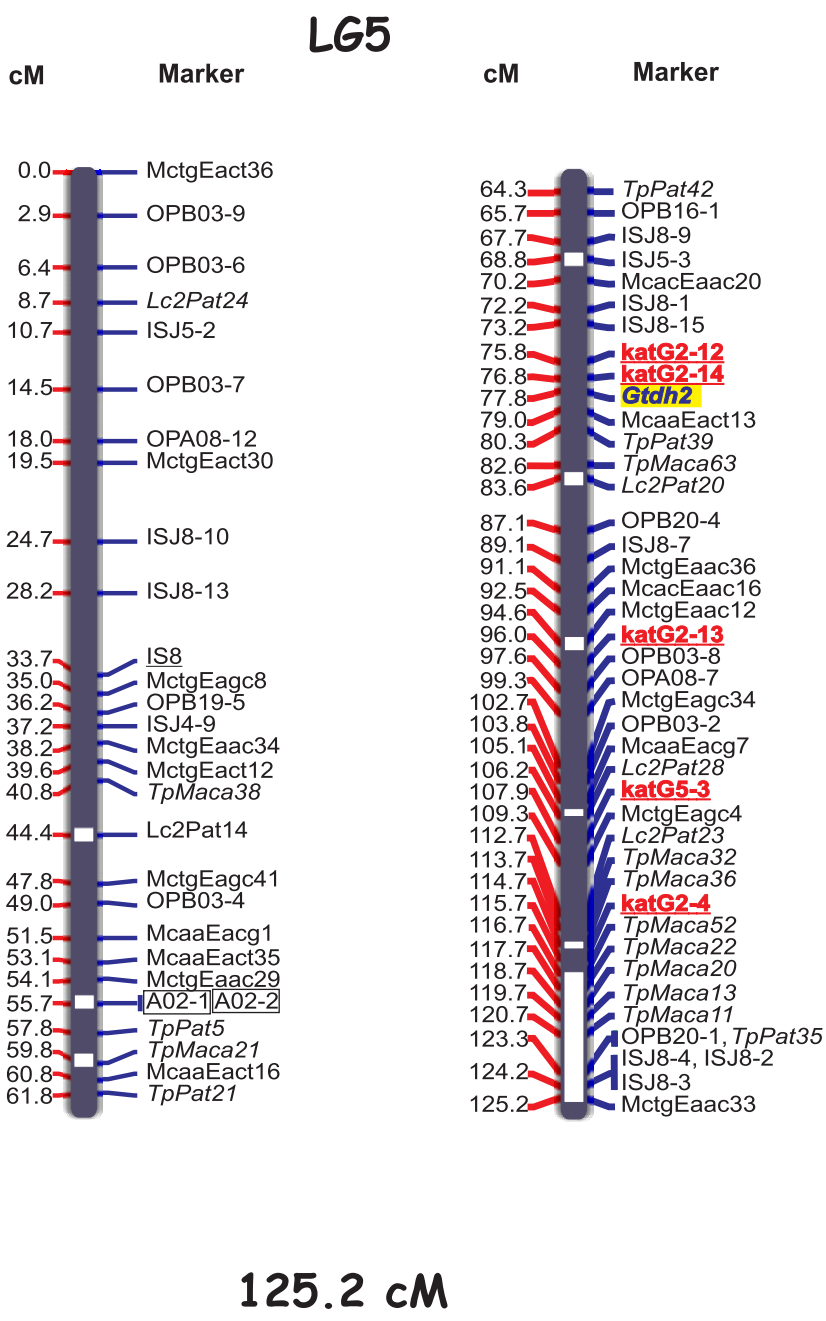


Figure 6.5E. Genetic linkage map of *L. multiflorum* and *L. perenne* based on 502 morphological, enzyme and DNA markers – linkage group LG5

- Shadow boxes, bold, italic – enzymatic loci, bracket – SSR loci, italic – transposon loci (Tp – *Tpo1* specific, Lc2 – *Lolcopia2* specific), bold, underlined – B-SAP loci,
- White boxes on chromosomes – regions with disturbed segregation

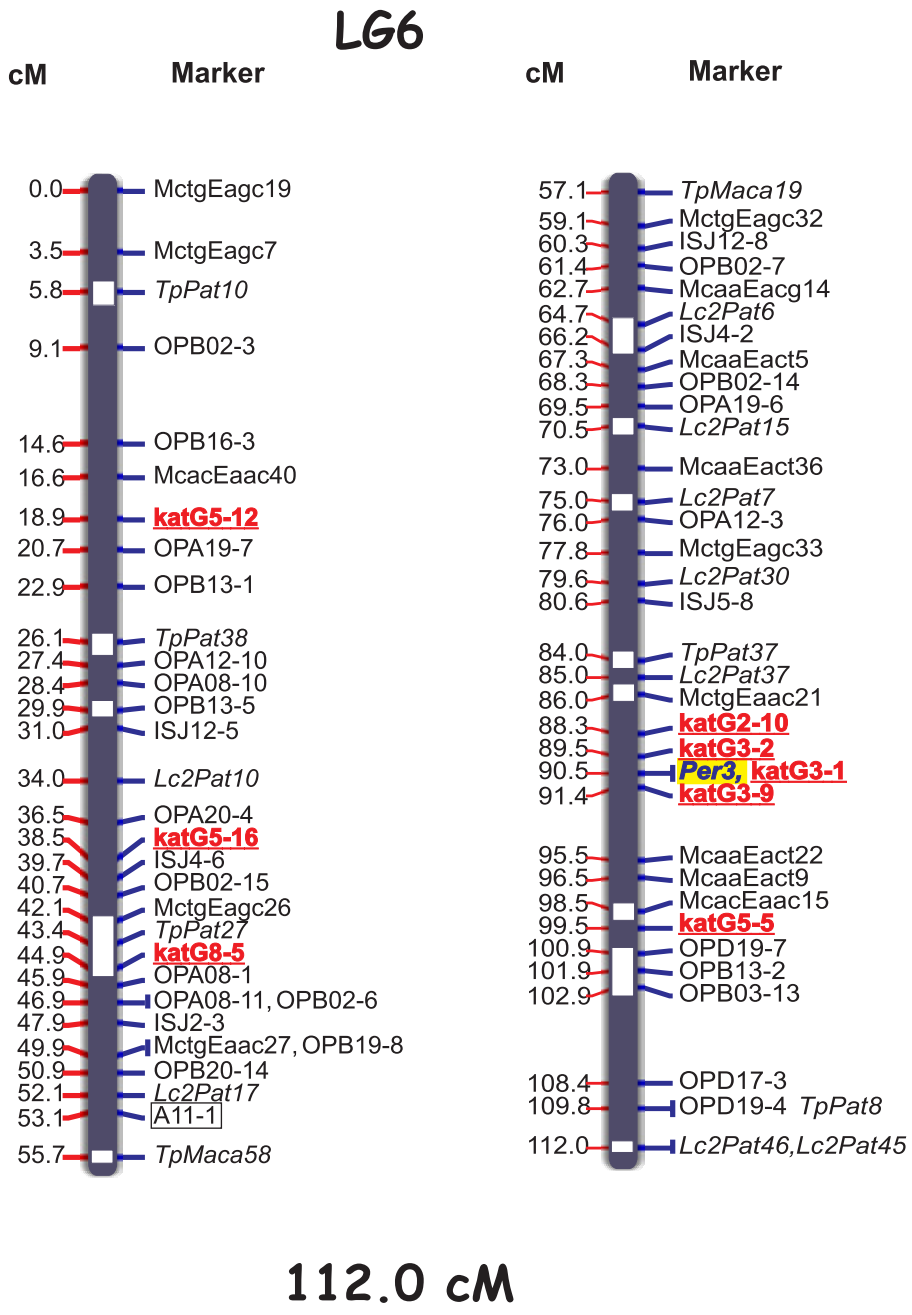


Figure 6.5F. Genetic linkage map of *L. multiflorum* and *L. perenne* based on 502 morphological, enzyme and DNA markers – linkage group LG6

- Shadow boxes, bold, italic – enzymatic loci, bracket – SSR loci, italic – transposon loci (Tp – *Tpo1* specific, Lc2 – *Lolcopia2* specific), bold, underlined – B-SAP loci,
- White boxes on chromosomes – regions with disturbed segregation

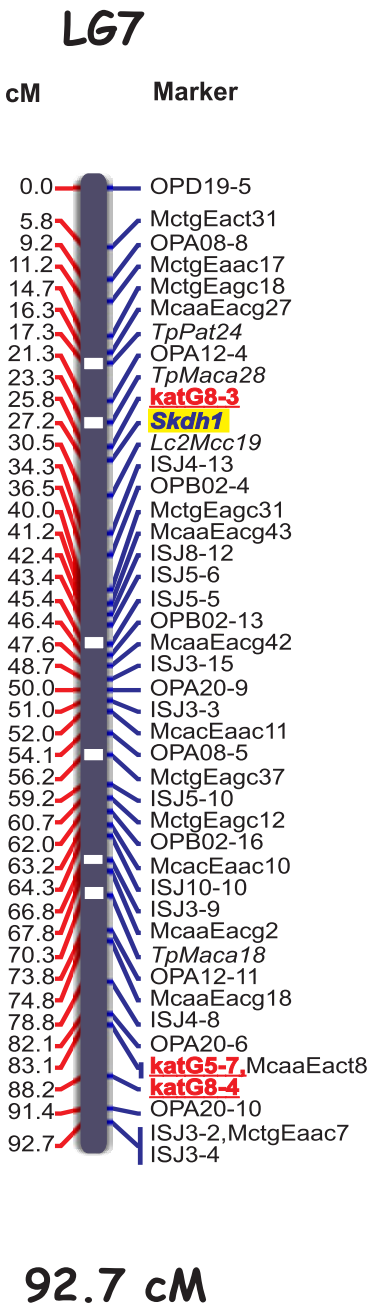


Figure 6.5G. Genetic linkage map of *L. multiflorum* and *L. perenne* based on 502 morphological, enzyme and DNA markers – linkage group LG7

- Shadow boxes, bold, italic – enzymatic loci, bracket – SSR loci, italic – transposon loci (Tp – *Tpo1* specific, Lc2 – *Lolcopia2* specific), bold, underlined – B-SAP loci,
- White boxes on chromosomes – regions with disturbed segregation

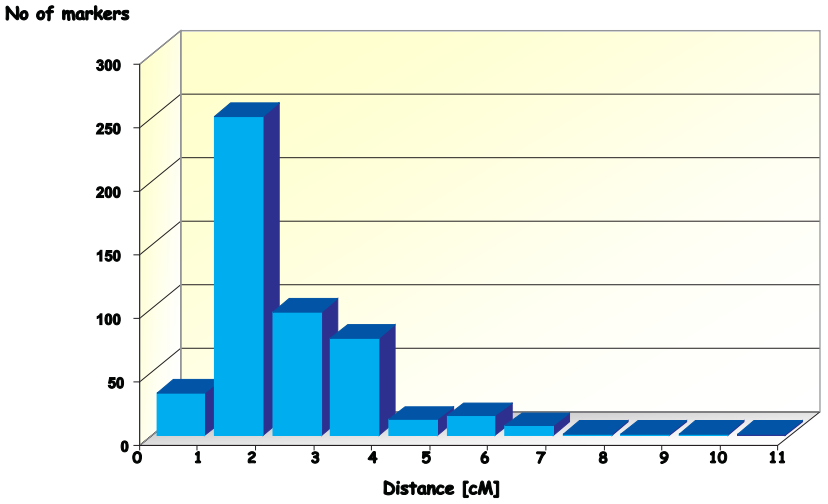


Figure 6.6. Histogram of distance between adjacent markers on the linkage map of *L. multiflorum* and *L. perenne*

The vast of different DNA markers enabled to construct the linkage map with the quite uniform distribution of markers and good coverage of the whole map distance. The only one gap of 10 cM was observed and none big cluster of markers was found. Although several small clusters of 3-4 markers could be observed the gaps between them were successfully filled by the other types of markers. A nice example included two small clusters of AFLP markers located between 45.7 cM and 60.9 cM on LG2 (Figure 6.5B), however a gap between them was filled by the RAPD marker, OPB20-6, and ISJ marker ISJ4-7.

Surprisingly, RAPD and ISJ markers showed the most uniform distribution between all linkage groups (Table 6.7). It is worthy to note that some clustering between AFLP and SSAP markers was expected because the former markers are mixture of SSR, transposon and restriction site markers. It was therefore, somehow unexpected that AFLPs tended to group rather with RAPDs than transposon-based markers. On the other hand transposon-based markers were not equally distributed between linkage groups. They were more frequent on LG2, LG4 and LG5 than on the other linkage groups. Markers specific to *Tpo1*, DNA transposon from the CACTA family, were clustered on the distance of 4 cM at the end of LG5. It should be also realized that katG markers revealed by primers complementary to the bacterial catalase-peroxidase gene were predominantly observed in close proximity to enzymatic loci.

6.3.3. Segregation distortions in *L. multiflorum* and *L. perenne* crosses

Segregation distortions in the interspecific mapping population, BR3 x NZ15

Genotyping of 269 F_2 individuals from the cross between two botanical taxa *L. multiflorum* and *L. perenne* did not reveal substantial non-Mendelian inheritance across the majority of DNA and enzymatic loci. Single-locus segregations were consisted with the expected 3:1

(or 1:2:1 for codominant loci) Mendelian ratio at around 74% of loci (Table 6.8). The remaining 26% of markers manifested significant deviations from the expected ratio. The distorted loci were rather equally split among all linkage groups and the percentage of skewed markers for each LG ranged around the overall value for the whole genome. Somehow more distorted loci were only located on LG3 and LG4 (37 and 33%, respectively). Similarly, the magnitude of distortions as indicated by the mean χ^2 statistics was comparable for all linkage groups. The LG5 was the only exception, for which the mean χ^2 statistics was twice as high as for other groups. This extreme value was primarily attributable to two linked, dominant SSR loci, (A02-1, A02-2), for which the observed ratio was 1:1 instead of 3:1. On opposite, the lowest number of distorted markers with the lowest χ^2 was typical for LG7. These not so severe distortions may be due to the fact that only few transposons were mapped on it.

Table 6.8. Markers showing distorted segregation in *L. multiflorum* and *L. perenne*

Parameter		Linkage group							
		LG1	LG2	LG3	LG4	LG5	LG6	LG7	Total
Skewed markers per marker type									
Enzymes	N°	2	1	0	1	0	0	0	**4**
	%[1]	40.0	33.3	0.0	20.0	0.0	0.0	0.0	**25[2]**
RAPD	N°	4	3	5	7	1	4	0	**24**
	%	21.0	11.1	38.5	25.9	8.3	17.4	0.0	**18.2**
ISJ	N°	3	6	4	5	4	1	3	**26**
	%	25.0	46.2	28.6	45.5	33.3	16.7	25.0	**32.5**
SSR	N°	1	0	0	0.0	2	0	0	**3**
	%	**50.0**	0.0	0.0	0.0	**100.0**	0.0	0.0	**50.0**
AFLP	N°	4	1	0	4	2	3	0	**14**
	%	13.3	3.7	0.0	19.0	10.5	21.4	0.0	**10.2**
SSAP- *Tpo1*	N°	1	6	3	6	8	5	3	**32**
	%	25.0	42.8	**100.0**	**54.5**	**53.3**	**71.4**	**100.0**	**56.1**
SSAP- *Lolcopia2*	N°	5	3	6	4	3	4	0	**25**
	%	**71.0**	**75.0**	**85.7**	**66.7**	**50.0**	44.4	0.0	**64.1**
B-SAP – IS6110	N°	1	0	0	0	0	0	0	**1**
	%	25.0	0.0	0.0	0.0	0.0	0.0	0.0	**16.7**
B-SAP - katG	N°	0	0	1	1	0	1	0	**3**
	%	0.0	0.0	25.0	20.0	0.0	12.5	0.0	**11.1**
Total skewed markers	N°	**21**	**20**	**19**	**28**	**20**	**18**	**6**	**132**
	%[3]	**24.4**	**21.9**	**36.5**	**32.6**	**27.8**	**26.1**	**13.0**	**26.4**
Mean χ^2 for skewed markers		20.5[b]	13.8[b]	15.1[b]	15.6[b]	42.4[a]	15.5[b]	11.3[b]	**20.0**
% in favour of P_1 (*L. multiflorum*)		14.3	60.0	36.8	46.4	65.0	50.0	66.7	**46.2**
% in favour of P_2 (*L. perenne*)		80.9	40.0	63.2	53.6	35.0	50.0	33.3	**53.0**

[1]In comparison with total number of markers of a given kind mapped on LG;
[2]in comparison with total number of markers of a given kind mapped;
[3]in comparison with total number of markers mapped on a given LG
[ab]Different letters mean significant differences between mean $\chi 2$ for each LG at P=0.05 for LSD test

The direction of distortion i.e., in favour of the female parent, *L. multiflorum* (P_1) or male parent, *L. perenne* (P_2) depended on a linkage group. Such selection was observed for loci mapped on five linkage groups. In a case of three groups, LG2, LG5 and LG7, *L. multiflorum* alleles were favoured whereas the opposite trend was demonstrated by the majority of loci mapped on LG1 and LG3. For these loci, the excess of individuals of the male parent phenotype, *L. perenne* was present in the F_2 population. By contrary, markers showing non-Mendelian inheritance and located on LG4 and LG6 were equally split between deviation towards *L. multiflorum* and *L. perenne*.

When distorted markers were analysed within the context of their positions along the linkage groups, it became obvious that genomic regions of distortions were distributed throughout the whole genome without a visible tendency to clustering (Figure 6.5A-G). In contrast, distorted regions seemed to be rather correlated with a marker type than with the specific position within the genome. An early glimpse on the linkage map allowed deducing that the majority of distortions were related with the presence of transposons. This was emphasized the best at the distal part of the linkage group LG5 (Figure 6.5E), where the distorted region of 10 cM containing *Tpo1* transposons of non-Mendelian inheritance was located. Several RAPD, ISJ and AFLP markers were mapped nearby and they were also distorted, probably because of the *Tpo1* neighbourhood.

There were additional reasons why transposons should be regarded as major forces responsible for the birth of distorted regions. More than 50% of *Tpo1* and more than 60% of *Lolcopia2* insertion sites around the whole genome exhibited extreme deviations from the expected 3:1 segregation (Table 6.8). Vastly greater accumulation of distorted transposons was visible if a particular linkage group was taken into account. The most dramatic findings came from LG3 were no *Lolcopia2* and hardly any *Tpo1* mobile elements were inherited in the Mendelian fashion. It was also true that the other markers with non-Mendelian inheritance were usually linked with transposons. Examples involved the distorted AFLP marker, McacEaac29 on LG2 lying within 2.8 cM from *Lolcopia2*, a group of ISJ, RAPD and *katG* loci on LG3 flanked by *Lolcopia2* retrotransposon and *Tpo1* DNA transposon and many others. Another interesting feature of transposon distortions was the predominant selection towards the *L. perenne* parent (Table 6.9) i.e., the parent of which low number of insertion sites was typical. Noteworthy, an excess of *L. multiflorum* phenotypes in F_2 was observed only if the female parent did not posses a band. To check if selection was against the insertion of transposons, distortions were considered with regard to the presence or absence of bands. And it became clear that F_2 homozygous individuals not carrying a transposon in a given insertion site were favoured. Not unsurprisingly, the selection towards the lack of a band was also observed for those few AFLP markers that were distorted (Table 6.8-6.9). It is common that AFLP primers also reveal transposons. Furthermore, many transposons have been isolated from AFLP gels. It could be speculated therefore, that distorted AFLP markers were in fact transposon loci. The same could be true for the only distorted locus revealed by primers complementary to *M. tuberculosis* IS6110 sequence, which is indeed a mobile element. Among other DNA markers, significant deviations were found for half of SSR loci (H06 on LG1, A02-1 and A02-2 on LG5). However, these data could be biased due to a small sample of SSR loci analysed. Second, it could not be excluded that they also represented transposon sequences all the more they were linked with *Lolcopia2* or *Tpo1*.

Whether selection against a band was typical of transposons or not was proved indirectly by distortion patterns revealed by such markers as ISJ and RAPD. The former exhibited a little raised level of distortions in comparison with the overall mean (33% vs. 26%), but on opposite to transposons, the presence of a band was favoured (Table 6.9). The distorted RAPD loci were equally split between deviations towards the presence and absence of a band. Noteworthy, a relatively few RAPD markers segregated in the non-Mendelian fashion (18%). Another example of opposite to transposon selection was provided by katG markers.

Table 6.9. Magnitude of segregation distortions for different types of markers

Marker type	mean χ^2	In favour of P_1 *L. multiflorum* [%]	In favour of P_2 *L. perenne* [%]	In favour of presence of a band [%]	In favour of absence of a band [%]
Enzymes	20.5[b]	25.0	75.0	50.0 (fast)	25.0 (slow)
RAPD	14.3[c]	66.7	33.3	45.9	54.2
ISJ	18.9[bc]	69.2	30.8	61.5	38.5
SSR	63.9[a]	0.0	66.3	0.0	66.3
AFLP	14.8[c]	14.3	85.7	14.3	85.7
SSAP-*Tpo1*	23.4[b]	37.5	62.5	28.0	72.0
SSAP-*Lolcopia2*	21.8[b]	40.0	60.0	15.6	84.3
B-SAP- IS6110	10.3[c]	0.0	100.0	0.0	100.0
B-SAP- katG	9.2[c]	0.0	100.0	100.0	0.0

[ab]Different letters mean significant differences between mean χ2 for each marker type at P=0.05 for LSD test

In general, these markers segregated in the expected 3:1 ratio, and only three of them were skewed, all towards the presence of a band.

Overall, with regard to observed magnitudes of DNA marker distortions, data from the *L. multiflorum* x *L. perenne* mapping population documented a relatively little genetic incompatibilities between both taxa. The majority of markers segregated in the Mendelian fashion, and distortions were mainly attributable to transposons.

Segregation of enzymatic markers in intra- and interspecific crosses

Whether the observed level of marker distortions in the interspecific population, BR3 x NZ15 was low or not the comparisons were made between this population, another interspecific population, HU5 x BO2, and two intraspecific populations representing *L. multiflorum* and *L. perenne*. However, to maintain a realistic size of experiment, only enzymatic markers were used.

Surprisingly, the proportion of distorted enzymatic loci did not differ in inter- and intraspecific crosses. In all populations single-locus segregations were consisted with the expected 1:2:1 ratio or 3:1 ratio in a case of null alleles at about 70% of loci (Table 6.10). Significant segregation distortions were indicated by chi-square analysis for six loci including *Est2*, *Est4*, *Mdhp2*, *Per1*, *Pgdh1* and *Sod1*. The highest fraction of distorted markers (50%) was observed in the intraspecific cross of *L. multiflorum* whereas no distorted segregation was found in the interspecific cross between *L. perenne* (HU5) and *L. multiflorum* (BO2). In the remaining two crosses (BR3 x NZ15; KY20 x BB6) significant segregation distortions were indicated for 25% of loci. There was no tendency in favour of female or male parent alleles. However, the most obvious trend was an excess of heterozygotes at the majority of distorted loci such as *Per1* in the interspecific cross BR3 x NZ15, *Est2* in the intraspecific cross VA7 x AS17 and *Mdhp2* in both these crosses. This type of distortions was accompanied by an excess of fast alleles in the cross BR3 x NZ15. The opposite trend, a deficiency of heterozygotes, was found at *Pgdh1* in the BR3 x NZ15 population. The most extreme distortion as indicated by χ^2 was seen at the *Est2* locus in *L. multiflorum* population (VA7 x AS17) at which almost no slow alleles were observed and at *Sod1* in *L. multiflorum* x *L. perenne* population (BR3 x NZ15) that, in turn, was characterized by an ex-

Table 6.10. Chi-square significance test of enzymatic alleles segregating in four F_2 populations of *L. multiflorum* and *L. perenne*

Allel	Locus	BR3 x NZ15			HU5 x BO2			VA7 x AS17			KY20 x BB6		
		F	F/S	S	F	F/S	S	F	F/S	S	F	F/S	S
Acoh1	N	69	120	80	44	106	51	homozygous			homozygous		
	χ^2 1:2:1	4.03			1.09								
Adh	N	67	144	58	homozygous			homozygous			11	23	10
	χ^2 1:2:1	1.94									0.14		
Aat2	N	69	131	69	49	101	51	48	106	51	17	16	11
	χ^2 1:2:1	0.18			0.04			0.33			4.91		
Cap2	N	69	133	67	homozygous			homozygous			homozygous		
	χ^2 1:2:1	0.06											
Est1	N	homozygous			145		56	homozygous			homozygous		
	χ^2 3:1				0.88								
Est2	N	homozygous			homozygous			24	178	3	homozygous		
	χ^2 1:2:1							115.53***					
Est3	N	homozygous			homozygous			50	106	49	homozygous		
	χ^2 1:2:1							0.25					
Est4	N	71	124	74	51	98	52	homozygous			6	17	21
	χ^2 1:2:1	1.71			0.13						12.50**		
EST-flu3	N°	62	134	73	homozygous			homozygous			homozygous		
	χ^2 1:2:1	0.90											
Gtdh2	N°	64	133	72	homozygous			homozygous			homozygous		
	χ^2 1:2:1	0.51											
Hk	N	76	135	58	homozygous			homozygous			homozygous		
	χ^2 1:2:1	2.41											
Idh1	N	70	136	63	49	103	49	homozygous			homozygous		
	χ^2 1:2:1	0.40			0.12								
Mdh2	N	66	134	69	homozygous			homozygous			13	20	11
	χ^2 1:2:1	0.07									0.54		
Mdhp2	N	73	154	42	homozygous			37	119	49	homozygous		
	χ^2 1:2:1	12.80**						6.72*					
Per1	N	82	139	48	homozygous			homozygous			homozygous		
	χ^2 1:2:1	8.90*											
Per3	N	57	129	83	homozygous			homozygous			homozygous		
	χ^2 1:2:1	5.48											
Pgdh1	N	82	109	78	homozygous			homozygous			homozygous		
	χ^2 1:2:1	9.79**											
Skdh1	N	78	121	70	48	99	54	homozygous			homozygous		
	χ^2 1:2:1	3.19			0.40								
Sod1	N	66	86	117	homozygous			homozygous			homozygous		
	χ^2 1:2:1	54.32***											
Distorted markers		N°	4		N°	0		N°	2		N°	1	
		%	25		%	0		%	50		%	25	

*Significant deviation from 1:2:1 segregation at P=0.05, **at P=0.01, ***at P=0.001, F - fast allele, S - slow allele

cess of the slow allele. Interestingly, *Mdhp2* was the only locus, at which segregation was observed in two F_2 populations, in the interspecific, BR3 x NZ15, and intraspecific, VA7 x AS17, and in both of them frequencies of alleles deviated significantly from the Mendelian ratios.

Noteworthy, all enzymatic loci but *Sod1*, deviating from the Mendelian model of inheritance in the interspecific population BR3 x NZ15, were tightly linked with transposon sequences.

6.3.4. Inheritance of selected traits in intra- and interspecific crosses

Seedling root fluorescence (SRF)

Seedling root fluorescence (SRF) segregated in both interspecific F_2 populations (BR3 x NZ15 and HU5 x BO2) and in the F_2 population of *L. multiflorum*, VA7 x AS17. Unsurprisingly, no segregation was observed in the *L. perenne* F_2 population, KY20 x BB6, which was derived from nonfluorescent parents (Table 6.11). The roots of the F_1 hybrid did not fluoresce as well.

Segregation of seedling root fluorescence was consisted with the monogenic dominant mode of inheritance in the *L. multiflorum* population and in the F_2 derived from the inter-

Table 6.11. Segregation of seedling root fluorescence in F_2 populations of *L. multiflorum* and *L. perenne*

Type of a cross	Cross combination	Phenotypes					Expected ratio in F_2 (F:NF)	χ^2
		P_1	P_2	F_1	N° of F_2 plants: F	N° of F_2 plants: NF		
L. multiflorum x L. perenne	**BR3 x NZ15**	F	NF	F	154	115	3:1	45.21***
							9:7	0.11
L. perenne x L. multiflorum	**HU5 x BO2**	NF	F	F	157	44	3:1	1.04
L. multiflorum x L. multiflorum	**VA7 x AS17**	F	NF	F	148	57	3:1	0.86
L. perenne x L. perenne	**KY20 x BB6**	NF	NF	NF	0	44	-	-

F – fluorescent plants, NF – nonfluorescent plants; ***Significant deviation from the expected ratio at P=0.0001

specific cross between *L. perenne* and *L. multiflorum*, HU5 x BO2. However, the significant deviation from the expected 3:1 ratio was observed in the second interspecific population derived from the cross between *L. multiflorum* and *L. perenne*, BR3 x NZ15. By contrary, the segregation of seedling root fluorescence in this population was in agreement with the 9:7 ratio typical of two complementary genes.

A report from genetic mapping studies in the *L. multiflorum* x *L. perenne* population, BR3 x NZ15 provided other evidences that at least two genes may be responsible for the SRF character. Two regions were identified on the first linkage group (Figure 6.5A). The first locus was located on the proximal part of LG1 and it was tightly linked with *Lolcopia2* marker Lc2Pat22 (1.3 cM). It was also located more than 30 cM away from *Est4* locus. This locus was further assigned as *SRF1* with respective alleles, *SRF1* as dominant and *srf1* as recessive. The second one, further described as *SRF2* with dominant *SRF2* and recessive *srf2* alleles, mapped on the same linkage group but in the distal part, in about 3.4 cM away from of a small cluster of three AFLP markers, McacEaac32, McaaEact15 and MctgEaac22. In proximity to this region, the insertion site of *Tpo1* transposon (TpPat41) was found and enzymatic locus, *Pgdh1* encoding phosphogluconate dehydrogenase (within 9 cM). Appar-

ently, the presence of two *SRF* loci in the BR3 x NZ15 population agreed well with the data from the other crosses. The HU x BO2 F_2 population identified the *SRF* locus tightly linked to *Est4*; hence this locus corresponded to *SRF1* identified in the BR3 x NZ15 population. In the *L. multiflorum* population (VA7 x AS17) the seedling root fluorescence was mapped about 24 cM from *Aat2* locus. Thus this should be *SRF2* that indeed was located about 30 cM apart from Aat2 locus on the genetic map.

Enzymes

The high rate of allozyme polymorphism in the majority of populations made it possible to determine map location for a number of enzyme genes (Figure 6.7). A total of 16 markers were located on the linkage map of *L. multiflorum* x *L. perenne* (Figure 6.5). They were present over six linkage groups with the tendency to grouping on LG1 and LG4. Five enzymatic loci were found on each of them. The first linkage group (LG1) included following isozyme loci, *Est4*, *Est-flu3*, *Aat2*, *Sod1*, and *Pgdh1* spanned over 120 cM (Figure 6.5A). In addition linkage between Est3 and Aat2 was observed in the population of *L. multiflorum*, VA7 x AS17. The distance between both loci was 18.0 cM, thus it was nearly the same as the distance between *Est3-flu3* and *Aat2* on the linkage map (20 cM). It is likely, from the location, that two markers described as *Est3* and *Est-flu3* and disclosed by different staining procedures, probably represented the same locus.

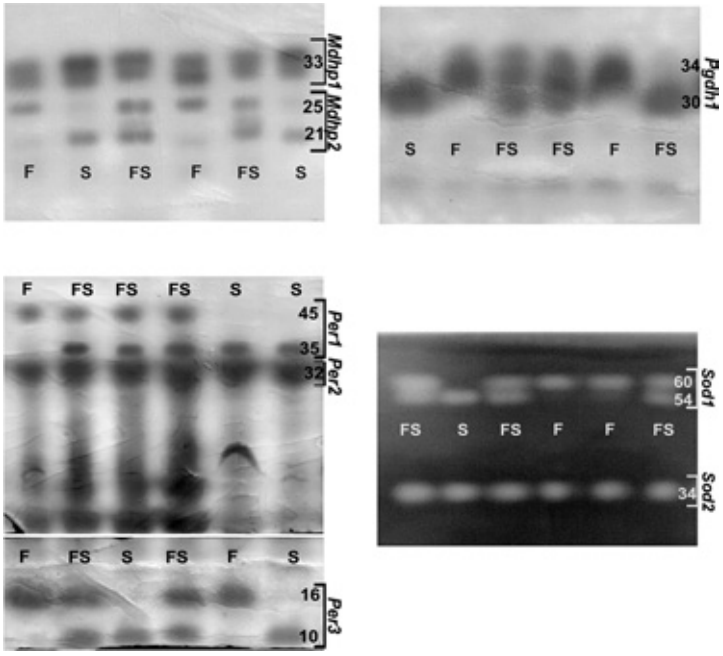


Figure 6.7. Segregation of isoenzymes in the F_2 population from the cross between *L. multiflorum* and *L. perenne*, BR3×NZ15
(F – homozygote with fast allele, S – homozygote with slow allele, FS – heterozygote)

Another five enzymatic loci (*Mdh*2, *Per1, Adh1, Idh1* and *Acoh1*) mapped on LG4 and covered around 40 cM (Figure 6.5D). The linkage between genes encoding aconitase hydratase (*Acoh1*) and isocitrate dehydrogenase was observed in the HU5 x BO2 interspecific family as well. Three enzymatic loci, *Cap*2, *Mdhp*2 and *Hk1* spanned over the distance of 70 cM in the middle of LG2 (Figure 6.5B). The locus *Est*2 should be also placed within this group for the reason that it was moderately linked with *Mdhp*2 in the population of *L. multiflorum* VA7 x AS17. The remaining three enzymatic loci segregating in the BR3 x NZ15 were allocated to three linkage groups; *Gtdh*2 to LG5, *Per*3 to LG6, and *Skdh1* to LG7 (Figure 6.5E-G). The common feature of these enzymatic loci was linkage with katG markers and proximity to transposons.

Bacteria Specific Amplification Polymorphism (B-SAP)

In total 33 markers revealed by primers complementary to different *M. tuberculosis* sequences were mapped on the *L. multiflorum* x *L. perenne* genetic map that supported the usefulness of such markers in genetic studies of plants. Moreover, the majority of them segregated in the Mendelian fashion in the mapping population, BR3 x NZ15. Significant distortions were observed for four markers, hence for 12% of loci and this value was significantly below the overall level observed in this cross combination. Because these markers were derived from bacteria, they were further referred to as Bacteria Specific Amplification Polymorphism (B-SAP) with adding the name of a sequence or a gene from which primers were derived if necessary. Thus markers revealed by primers complementary to *KatG* gene were assigned as katG and those revealed by primers complementary to IS6110 as IS markers.

The majority of IS markers from six mapped were found on LG1. Two of them were located on the proximal part while the other two were completely linked with OPA08-16 marker on the distal part of this group. Another marker, IS1 was linked with *Tpo1* transposon (TpMaca41a) on LG2, while IS8 was linked with AFLP marker, MctgEagc8 on LG5.

The katG markers were distributed among seven linkage groups. The most of them were allocated on LG6 (eight) followed by LG4 and LG5 (five on each group). The most striking result was the tight linkage between several katG markers on LG6 with locus *Per3* encoding peroxidase. This result confirmed our previous predictions that at least some katG markers can amplify peroxidase loci in plants. Similarly, another locus responsible for peroxidase, *Per1* located on LG4 was also linked with katG markers. Noteworthy, except of peroxidases, katG markers tended to be linked with other enzymatic loci, especially encoding dehydrogenases. For example, katG3-7 on LG2 was linked with *Hk1* encoding hexokinase, katG5-9 on LG4 was linked with *Mdh*2 encoding NAD-depended malate dehydrogenase, two markers, katG8-6 and katG3-8 with *Acoh1* responsible for aconitase hydratase and katG2-12 and katG2-14 with *Gtdh* encoding glutamate dehydrogenase. Moreover a linkage between *Est4* and katG5-11 was observed on LG1.

The katG markers segregated in the Mendelian fashion according to dominant mode 3:1. They were rarely distorted. However, all distorted katG markers were selected towards the presence of a band.

6.4. DISCUSSION

6.4.1. At what stage of speciation are *L. multiflorum* and *L. perenne*?

Taxonomists very often classify species based on one or two visible morphological differences. The idea behind this is quite simple. Species that are separated by reproductive isolation also differ by more "ordinary" morphological characters that play no necessary role in blocking gene flow (Orr 2001). Despite the fact that reproductive barriers and morphological differences are often connected, there is a good reason to think that this association is partly casual. Cryptic species that are morphologically very alike, yet reproductively isolated are good examples that evolution is not necessarily correlated with phenotypic diversification. By contrary, phenotypic diversification does not always go hand-in-hand with a birth of reproductive barriers. Huge mutant collections of crop species are the best examples of "ordinary" morphological differences that are not related with speciation. On the other hand, speciation is gradual and it is somehow uncoupled from processes of intraspecific population differentiation. It is a challenging task to find out whether observed differentiation is coupled or not with speciation especially at the very early stages at which no reproductive barriers exist or eventually their birth can be hardly observed. *L. multiflorum* and *L. perenne* belong to these "troublesome" species. One, the most traditional approach to study the reproductive barrier formation often involves the analysis of intra- and interspecific hybrids with respect to fertility. The easy of hybridisation and the lack of any signs of hybrid sterility coupled with possibility of backcrossing observed in all analysed *Lolium* crosses states for the lack of reproductive barriers between incipient species. Obviously, species that are fully interfertile can not be classified as biological species, however crossability does not exclude that they are diversifying and that the birth of a reproductive barrier can be observed only in certain loci. The difficulties are that black and white situations are rare and very often, even hybrids between cultivars exhibit some level of sterility due to different mechanical damages during artificial crossing. Furthermore, a lesson learnt from maize has shown that fully interfertile species in crossing experiments, may be reproductively isolated in nature through the action of a single allele, *Tcb1-s* differing in frequency in species under question (Kermicle 2006). In *L. multiflorum* and *L. perenne* at least two self-incompatibility genes were identified and they may play a role in limiting the recombination frequency at certain loci and distorting segregation of adjacent markers (Thorogood et al. 2005). Thus, the crucial distinction in a case of *L. multiflorum* and *L. perenne* is not whether a reproductive barrier exists (obviously it does not), but whether or not they are undergoing speciation, and more exactly if any signs of a species boundary can be found. Another traditional method to study it involves the comparison of the level of polymorphism in the crosses between related species that could be hybridized and distribution of polymorphic markers between parents. It is believed that parents belonging to different species have fixed different alleles in many loci thus they differ in so called multilocus genotypes. Such differences have been observed in *L. multiflorum* and *L. perenne* at several enzymatic loci, but - surprisingly in intraspecific crosses. Conversely, enzymatic and DNA alleles in interspecific crosses were equally split between parents. Thus, there is another reason to throw away the thesis about species differences between *L. multiflorum* and *L. perenne*.

Until recently the processes at the earliest stages of evolution have been black boxes. Over the past several years, the ready availability of genome sequences, molecular markers and maps allows to address questions about early evolutionary processes in much greater depth than ever before, since the role of variation at individual loci can be assessed. One of the most common observations when two species are crossed is that some molecular markers do not segregate according to the expected Mendelian fashion. The degree of distortion is thought to be positively correlated with the level of divergence between taxa and generally, fewer alleles are distorted in conspecific crosses. In *Helianthus*, 7-13% of loci are distorted in intraspecific mapping populations compared to 23-90% of loci in interspecific crosses (Rieseberg et al. 2000). Although deviations from expected Mendelian ratios are commonly observed both within a species and between species, this phenomenon is often associated with the birth of reproductive barriers. From 34% to 42% of distorted markers in the interspecific cross between *L. multiflorum* and *L. perenne* observed by Warnke et al. (2004) seem to confirm the presence of species specific barriers between both taxa. It has been reasonable, therefore, to expect greater marker distortions in interspecific crosses analysed in the present studies in comparison with intraspecific ones. Again more surprises about *L. multiflorum* and *L. perenne* are emerging. The non-Mendelian inheritance was observed at about 50% of enzymatic loci in the *L. multiflorum* F_2 population but only at 25% of such loci in the interspecific population, BR3 x NZ15. Furthermore, none marker deviates from the expected ratio in another population derived from a cross between *L. perenne* and *L. multiflorum* (HU5 x BO2). These data inevitable entail that no barriers exist between both species and results of Warnke et al. (2004) are presumably more correlated with genetic background of parents and the mode of crossing. It should be stressed that all populations used in the present work were F_2 populations derived through crossing two plants and selfing F_1 what significantly simplifies segregation analyses in comparison with three-generation or two-way pseudotestcross populations used in the other studies of *Lolium*.

Particularly intriguing is the high level of distortions at enzymatic loci in *L. multiflorum* populations suggesting genomic incompatibilities between parents. It originated from a cross between an ecotype and a cultivar. It can be hypothesized, therefore, that the cultivar has been considerably changed during breeding. This finding is in agreement with the earlier results (Chapter 3-4) demonstrating that breeding activities and presumably introgression of some genes from *Festuca* have resulted in substantial genomic rearrangements in modern *L. multiflorum* cultivars. Another likely biological explanation involves the action of self-incompatibility locus. Both distorted enzymatic loci are linked and lie on LG2. This group corresponds to LG2 of Hayward et al. (1998), on which there is evidence of the Z locus responsible for gametophytic incompatibility system. This locus is complementary to S locus located on LG1 and both S and Z alleles must be matched in pollen and style for an incompatibility reaction to occur (Thorogood et al. 2002). Both loci are highly polyallelic and show conserved synteny with the equivalent loci in *Secale cereal*e (rye). Such interpretations suggest that the non-Mendelian inheritance in *Lolium* is more attributable to the species level. The probability that two alleles match is obviously lower in interspecific crosses and thus, the distortions are less frequent, what is elegantly emphasized by the lower or lack of deviations from the expected ratios in two interspecific populations, BR3 x NZ15 and HU5 x BO2. The biological sense of self-incompatibility lies in the prevention from self-pollination and in-

breeding that can otherwise lead to expression of deleterious alleles. Indirectly it means that strong incompatibility reaction within *L. multiflorum* should promote crossing with *L. perenne*. Clearly, this interpretation stands out against the thesis that *L. multiflorum* and *L. perenne* are different species.

The DNA data from the mapping population between *L. multiflorum* and *L. perenne* (BR3 x NZ15) fully confirm the enzymatic outcomes. In total 26% of DNA markers are distorted in the *L. multiflorum* x *L. perenne* mapping population in comparison with 25% of enzymatic loci. The same proportion of disturbed segregation (20%) has been found in the first interspecific mapping population constructed by Hayward et al. (1998). The level of DNA marker distortions observed in the *L. multiflorum* x *L. perenne* population in the current studies is typical of intraspecific crosses as indicated by 22% AFLP and RFLP markers distorted in *L. perenne* mapping population, p150/112 (Jones et al. 2002b). From these comparisons one notable conclusion is that very little genomic incompatibilities exist between *L. multiflorum* and *L. perenne*. If this interpretation is correct it provides strong evidence of none reduction in recombination in F_1 hybrids, thus confirming the earlier studies on chromosome pairing (Naylor 1960).

Nevertheless, a case in point involves that some distortions at DNA loci are observed in *L. multiflorum* and *L. perenne*. Any deviation from Mendelian segregation ratios is an important feature that prevents the free exchange of genes and very often is attributable to a birth of reproductive barriers. Unequal representation of parental alleles in a progeny population appears to be related with unequal representation of alleles in gametes, differential survival or fertilisation success of haploid gametes, and differential survival of the diploid zygotes (Fishman and Willis 2005). Although the dominant mode of inheritance of the markers used for the map construction does not allow discriminating among the potential causes of distortions, it is unquestionable that they have resulted from various mechanisms at the genome level. Structural rearrangements in chromosomes are between the most important and frequent contributors to non-Mendelian inheritance in interspecific crosses (Rieseberg et al. 1995). First, duplications, inversions and translocations in the heterozygous stage lead to reduced pairing of chromosomes during the first meiotic division in F_1 hybrids. This inevitable entails unbalanced gametes with additional copies of genes or/and without some genes. If these aberrant gametes fuse the resultant zygotes are non-functional and hybrid sterility or semi-sterility is observed. At the genetic map level it is observed as large distorted regions on particular chromosomes. Results from *L. multiflorum* and *L. perenne* appear not to be consistent with the above mechanism. Distorted regions are rather equally distributed between all linkage groups. About 22-33% of markers are distorted on each linkage group with the only exception of LG7, where 13% of markers deviate from the expected ratios. However, even in this case all regions were scattered throughout the whole length of this group. A second rationale for rejecting the chromosomal alteration hypothesis is the map length of 1 014 cM. In general structural rearrangements have the effect of reducing map lengths. Meantime, the present map is comparable or even longer than all other published maps of *Lolium* (Table 6.12). It is also comparable with the length of the genetic maps of barley (GrainGenes 2007), whose genome size is very alike *Lolium*.

On the molecular level deviations from expected Mendelian segregation ratios are often attributed to the effects of genic interaction. The widespread distributions of distorted regions

throughout the genome of *L. multiflorum* and *L. perenne* all indicate that distortions are connected with particular markers, hence confirming the role of genic interactions. Changes in gene regulations or negative epistatic interactions between divergent alleles at different loci may reduce fitness and embryos carrying them are more likely to be aborted (Harushima et al. 2001). Conversely, positive interactions are responsible for increased fitness and greater proportion of embryos carrying them. Distorted RAPD markers indicate both types of interactions owing to equal representation of markers selected toward the presence of a band as well as the lack of it. Another noteworthy example includes positive selection towards the presence of a band at three distorted *katG* loci. Because katG markers are predominantly linked to enzymatic loci it is plausible that the selection towards a band means the selection towards a specific enzyme. For instance katG5-9 is tightly linked to *Mdh*2 encoding malate dehydrogenase. Concepts that protein variation is largely adaptive and selected by different environmental conditions are well documented in plants (Nevo et al. 1983). The same sort of interactions can be proposed for ISJ markers. Because they are specific to junctions between exons and introns they likely mark genes, among which some increase fitness. That is why they are predominantly selected towards the presence of a band. The examples of katG and ISJ markers illustrate the kinds of positive genic interactions that can act at the species level. They are not so severe in a sense that not so many markers are distorted.

At the opposite extreme, more than half of transposon markers are distorted. Indeed, transposons are prevalent in all distorted regions on *L. multiflorum* x *L. perenne* genetic map. Although, having in mind the results from the genetic diversity studies, it could be expected, the magnitude of distortion inevitable demonstrates the unprecedented power of mobile elements in driving the evolutionary processes. Importantly, nearly all transposon markers were selected against the presence of a band. In other words, individuals without insertions were favoured in the interspecific F_2 population. Each genome represents a compromise between transposon movement and the mechanisms of their immobilisation. Transposon activity may differ between closely related lineages leading to differences in accumulation of retrotransposons blocks between genes. Within these blocks retrotransposons are commonly nested by insertion into each other (Fedoroff 2002). Presumably, a higher number of insertion sites in *L. multiflorum* (Chapter 4) result from such differences in transposon activities. Transposons are known to be both segregation and post-segregation distorters. Many of them reduce the frequency of non-carrier individuals after fertilisation, the phenomenon opposite to that observed in the present studies. On the other hand, host genomes develop mechanisms that prevent the action of distortion driving elements. One possibility is the spread of unlinked genes that inhibit the selfish phenotype. Unlinked mutations that prevent the action of the driving element may spread in natural populations of a species and increase the number of non-carrier individuals. However, the distortion driving phenotypes will re-emerge in hybrids between species or even distant populations if one of the parents lacks repressor genes (Hurst and Werren 2001). If we turn again to *L. multiflorum* and *L. perenne* it is plausible that repressor genes present in both parents contribute to strong selection against insertion. Under this hypothesis the potential lack of the repressor genes in one of the parents would result in the rapid spread of transposons in the F_2 population and the individuals with transposons would be favoured. Because repressor genes evolve as an answer for the action of selfish elements specific to a species, the lack of such genes in a given population is regarded as

a sign of diversification. Taken together, the strong selection against insertions in F_2 population derived from a cross between *L. multiflorum* and *L. perenne* resulting from the presence of repression genes in both parents contributes to the lack of functional incompatibilities that would underlie speciation and birth of reproductive isolation. However, it can not be excluded that some of observed transposon distortions play an important role in genetic divergence and the route for speciation is still opened. The data indicate that the molecular clock for transposable elements is at least twofold more rapid than synonymous base substitutions within the genes (Ma and Bennetzen 2004).

6.4.2. Genetic map of *L. multiflorum* and *L. perenne*

A major question is to what extent the current set of markers covers the whole *Lolium* genome. The theoretical genome length is estimated to be 1 190 cM based on the mean chiasma frequency per chromosome of 1.7 observed in the interspecific cross of *L. perenne* x *L. multiflorum* (Naylor 1960). The map obtained here spans 1 014 cM and covers 85% of the whole genome. Thus, it is the most complete map among all published until recently. Their genome coverage ranges from 47% to 78% (Table 6.12). The average distance between markers of 2 cM also positively distinguishes the present map from all others *Lolium* maps with the most common distance about 3 cM (Jones et al. 2002a; Hirata et al. 2006).

Table 6.12. Comparison of map lengths in *L. multiflorum* and *L. perenne*

Species	Map length [cM] Genome coverage [%]	Markers	Authors
L. multiflorum x L. perenne	692 (58%)	RFLP, RAPD, enzymes	Hayward et al. 1998
L. multiflorum x L. perenne	712 (60%)	AFLP, RAPD, SSR, RFLP	Warnke et al. 2004
L. multiflorum x L. perenne	1 014 (85%)	SRF, Enzymes, RAPD, ISJ, SSR, AFLP, SSAP, B-SAP	Polok 2007
L. multiflorum	888 (75%)	SSR	Hirata et al. 2006
L. perenne	930 (78%0	AFLP	Bert et al. 1999
L. perenne	565 (47%)	RFLP	Armstead et al. 2002
L. perenne	811 (68%)	AFLP	Jones et al. 2002b
L. perenne	724 (61%)	SSR	Jones et al. 2002a

The first glimpse at the present map demonstrates that the most striking feature is the uniform distribution of markers between all linkage groups. Although some small AFLP or SSAP clusters can be observed, the gaps between them are filled by the other marker types. This is a considerable progress with all other *Lolium* maps, on which strong marker clustering is observed. For example SSR loci tend to group around the putative centromeric region on LG1 of the p150/112 map resulting in sub-optimal genome coverage (Jones et al. 2002a). Several SSR clusters are also reported on the *L. multiflorum* map, however it is not clear if they are related with centromers (Hirata et al. 2006). Similarly, large clusters are observed on AFLP maps (Bert et al. 1999; Jones at al. 2002b). The obvious outcome of marker clustering is the presence of gaps i.e., long parts of linkage groups without any marker. The maximum distance of 10.4 cM found on LG3 between ISJ12-1 marker and *Lolcopia2* specific marker, Lc2Pat18 is the only gap on the present *L. multiflorum* and *L. perenne* map. Indeed, it is twofold smaller in comparison with about 20 cM gaps observed

on AFLP or SSR maps. Furthermore, several gaps are usually found. For example, the AFLP map derived from p150/112 contains five gaps (Bert et al. 1999) while as many as five chromosomes are deficient of SSR markers on the map based on the F_2 population derived from contrasting cultivars of *L. perenne* (Gill et al. 2006).

Certainly the uniform distribution of markers on the present map resulted from the application of various techniques. These findings raise the more fundamental point that a well saturated genetic map can only be constructed based on several marker types. A vast of different markers ensures that various types of genomic sequences are matched. Undoubtedly, the application of transposon-based markers is one of the major improvements preventing from marker clustering. Successful transposable elements insert in both plant and animal species in the centric heterochromatin as well as into the others transposons. Although the former are tagged to some extent by AFLP markers, the latter are rarely recognised due to high accumulation of LTR sequences. However, both regions can be successfully filled by transposon based markers. The low efficiency of AFLP markers to discover clusters of transposons is indirectly confirmed by their more frequent clustering with RAPD markers than with transposons.

Difficulties in alignment of all maps based on a single p150/112 population but employing a single marker category in a given experiment illustrate well that high quality genetic map can only be produced if various markers are used at once. The most dramatic example includes the SSR and AFLP maps, both constructed in p150/112 by the same authors (Jones et al. 2002a; b) but both with different linkage group assigning and moreover any attempts to align both maps have not been undertaken. Similarly, at least two maps are based on the F_2 population derived from a cross between two cultivars of *L. perenne*, Perma and Aurora. The first employs RFLP (Armstead et al. 2002) while the second SSR (Gill et al. 2006). Unfortunately, no consensus map is created. Each map with different marker categories is surely advantageous over those mono-markers and it has greater utility both in QTL mapping and evolutionary studies. Even fewer markers ensure quite uniform genome coverage (Hayward et al. 1998; Warnke et al. 2004). Employment of different marker categories is of the highest importance if a map has to be used for evolutionary considerations. For example, among all markers employed, those based on transposons (SSAP) proved to be the most powerful tool for mapping of species boundaries. Strong directional distortions demonstrate the extent and nature of evolutionary processes that would be difficult to deduce from the other marker categories also because all others segregate according to the expected Mendelian ratios typical of intraspecific crosses. Furthermore, mapping of insertion sites demonstrates clearly that distorted regions are predominantly associated with transposons. Transposon markers are among the most unique features of the present maps.

6.4.3. Location of selected traits on the map of *L. multiflorum* and *L. perenne*

One of the most obvious outcomes of genetic mapping is the location of genes of interests. In comparison with major crops including rice, maize or barley, the knowledge about genetics of morphological characters in *Lolium* is scarce. Only recently some traits have been mapped including flowering time, self-incompatibility locus and few enzymatic loci (Yamada et al. 2005). However, existing information is relatively underdeveloped. The current genetic

maps based on anonymous markers are also of limited diagnostic value. However, it seems unrealistic to create the morphological maps in *Lolium* comparable to those of cereals owing to the lack of huge collections of morphological mutants, trisomics or translocation lines. One possible solution is to map expressed sequences that are correlated with functionally defined genes. Although the similarity with cereals supports the development of molecular markers based on orthologous loci, the current data demonstrate that it is no so simple task. All sequence specific tags used in the mapping studies prove to be monomorphic or difficulties in amplification of a specific band are encountered. It is not quite surprising. Similarly, sequence tagged site markers derived from *Medicago truncatula* are of low value in mapping a stiff-straw trait in *Pisum sativum* due to problems with specific amplification and polymorphism between parents (Polok K, unpublished data). It is reasonable to believe that common using of orthologous loci to tag specific traits in *Lolium* is the future. Hence, mapping of any morphological or enzymatic marker is of special values.

Among many characters, seedling root fluorescence commonly used for separation of annual from perennial ryegrass, belongs to the most important ones. The evidences of genetic basis of seedling root fluorescence (SRF) have been long postulated (Warnke et al. 2004), yet they are not well understood. The previous data (Chapter 5) demonstrating that SRF is encoded by at least two genes are confirmed by two unlinked loci, *SRF1* and *SRF2* mapped on LG1 as well as the segregation ratio of 9:7 typical of two complementary genes. Dominant alleles at both loci should be present in fluorescent plants. A second evidence for the two gene model comes from the other two cross combinations. The *SRF1* locus segregates in the interspecific population HU5-BO2 whereas SRF2 in the *L. multiflorum* population, VA7-AS17. If only one locus segregates, fluorescence is dominant over its lack. Both loci are moderately linked to enzymatic markers that can be used as alternative separators of annual from perennial ryegrass. However, a caution should be made that it is not tight linkage and the test will not be exact. Two loci responsible for seedling root fluorescence explain very well the difficulties in the stabilisation of the SRF level in cultivars of *L. perenne*. There is always a possibility that a dominant allele responsible for fluorescence is in one locus. As long as the second locus possesses two recessive alleles, the fluorescence is not observed. However, one can imagine that some plants from a given cultivar are recessive homozygotes at one locus e.g., *SRF1*, while they possess at least one dominant allele in another locus, *SRF*2. The respective genotypes could be written as follow, srf1srf1SRF2. The other plants have opposite genotypes i.e., at least one dominant allele at *SRF1* and they are recessive homozygotes at *SRF*2 (SRF1-srf2srf2). If these two types of plants are crossed the resultant zygote has at least 25% chances to have dominant genes at both loci (SRF1srf1SRF2srf2) and develop into a plant with fluorescent seedling roots.

Although isozyme loci are present on nearly all genetic maps of *Lolium*, none of them can be compared with the present with respect to their number. In total 16 loci are mapped based on the BR3 x NZ15 population in addition to two loci mapped in the others. To compare, the *L. perenne* x *L. multiflorum* genetic map of Hayward et al. (1998) consists of eight enzymatic loci, whereas on the others maps from one to three isozyme loci exist (Bert et al. 1999; Jones et al. 2002b, Warnke et al. 2004). The location of enzymatic loci on the present map is aligned with that of Hayward et al. (1998). The reasons for coupling of most isozyme loci on two lingage groups (LG1, LG4) are unknown. The plausible explanation is

that enzymes resolved by any protein electrophoresis are not a random sample but they are pre-selected based on easy detection by ordinary staining procedure. Notwithstanding these difficulties, enzymes are useful in the identification of linkage groups between many maps providing that sufficient number of enzymatic loci is employed. With 16 enzymatic loci it is possible to mark nearly all linkage groups. It is a pity that these rapid, reliable and cheap markers are so rarely used for map alignment.

An important outcome from the current map is the identification of some enzymatic loci for the first time. They include loci encoding aconitase hydratase, alcohol dehydrogenase, cytosol aminopeptidase, isocitrate dehydrogenase, malate dehydrogenases, peroxidases and shikimate dehydrogenase. In *Lolium*, similarly to other plants, two different isoenzymes oxidize L-malate, oxaloacetate and some other 2-hydroxydicarboxylic acid. The first enzyme (MDH) uses NAD as a cofactor while another NADP (MDHP). Two loci for both of them are identified by means of starch gel electrophoresis. Due to similarity of their zymograms, it was suspected that they may represent a single locus. Nevertheless, mapping studies demonstrated that these two enzymes are indeed encoded by two different loci allocated to different linkage groups, *Mdh*2 locus is mapped on LG3 whereas *Mdhp*2 locus on LG2.

Another spectacular achievement is that for the first time markers revealed by primers complementary to bacterial sequences are mapped (B-SAP). In spite of transposons, this is the most unique feature of the present map. Until now there has no such a map either in *Lolium* or in the other plants. Two types of bacteria-specific markers are located, IS markers revealed by primers complementary to IS6110 insertional element of *M. tuberculosis* and katG markers based on *KatG* gene of the same bacterium. Their utility in generation of species specific markers has been confirmed in several plant species (Zielinski and Polok 2005) however, there has not been clear what kind of sequences they are. It has been suggested that markers based on IS6110 can be related with transposon sequences whereas katG markers with peroxidases due to the fact that *KatG* gene encodes catalase-peroxidase. Nevertheless, this hypothesis remained to be corroborated in more direct genetic studies such as this. A counterproposal that can easily be expressed is a contamination of plant material by bacteria. In that case, most markers derived from bacterial sequences would segregate in a non-Mendelian fashion and would not be linked with any plant sequence. Consequently, they would be identified as solitary markers. Fortunately, the last theory is unlikely in the light of the Mendelian inheritance of nearly 90% of B-SAP markers and their allocation to linkage groups of *L. multiflorum* x *L. perenne*. Furthermore, their map position is not random, but they are predominantly linked with enzymatic loci, including both loci responsible for plant peroxidases.

The bacterial catalase-peroxidase belongs to the class I of peroxidases and as the only member of this family possesses two functions - it can oxidaze (catalase activity) and reduce hydrogen (peroxidase activity). Catalase-peroxidase of *M. tuberculosis* encoded by *KatG* gene is one of the most studied bacterial peroxidases due to its role in acquiring resistance to antibiotics. The gene is characterized by rather high variability and therefore, a set of primers derived from *KatG* gene is commonly used for strain identification. On the other hand, the plant peroxidases of class I originated from a common prokaryotic ancestor but they lost their catalase activity. The detailed Zamocky's (2004) studies revealed that they possess three corresponding areas involved in catalytic mechanism.

The region A is located on the distal side of the heme group and corresponds to the N-termini of bacterial catalase-peroxidase, while the regions B and C are located on proximal side of heme and correspond to the C-termini of bacterial enzyme. Furthermore, the CLUSTALX alignment of protein sequences of 60 plant peroxidases proved that the greatest sequence similarity (76%) is achieved on the distal side with the so called the catalytic triad (Arg92, Trp95, His96). Differences in the level of polymorphism revealed by different pairs of primers in *Calamagrostis arundinacea* and correlation between *katG* polymorphism and peroxidase zymotypes have provided the first evidences that at least some of katG primers can amplify sequences of plant peroxidases (Krzakowa et al. 2007). In general, the pairs of katG primers that reveal low polymorphism amplify the conservative region encoding the distal side of heme peroxidase while the relatively high polymorphism is generated by katG primers complementary to the sequence encoding the C-termini of *KatG* gene. The tight linkage between four katG markers and *Per*3 locus on LG6 of *L. multiflorum* and *L. perenne* as well as between katG2-15 and *Per1* on LG4 are among first and the strongest arguments that primers derived from *M. tuberculosis KatG* gene amplify sequences of plant peroxidases. This observation may have further applications in using katG markers as a road map for studying peroxidases in plants. Somehow surprising is that some katG markers are also linked with loci encoding the other enzymes such as *Gtdh1*, *Mdh*2, *Hk2* and *Acoh*. This can suggest that those enzymes are of bacterial origin as well.

The Mendelian inheritance and linkage with enzymatic loci together with earlier results from phylogenetic analyses (Zielinski and Polok 2005) strongly support the utility of katG markers derived from bacterial sequences in genetic studies in plants. The same is true for IS6110 based markers and we believe that for several other markers derived from bacterial sequences e.g., hot fragment of *rpo* and *pol* genes that have been also tested. Therefore we propose to assign this system as **Bacteria Specific Amplification Polymorphism (B-SAP)**. The main feature of all bacteria derived sequences is their relatively high conservatism within a species exhibited as low polymorphism and dramatic differences between even closely related species.

To summarize, the outcome of genetic mapping studies in *L. multiflorum* and *L. perenne* demonstrated clearly that no species boundaries exist between them. Eventually some diversification can be observed on transposon level but this is far away from the birth of reproduction barriers. Therefore the classification of both taxa as a single “species complex”, with subspecies, as already suggested in the preceding chapters, seems more appropriate to their biological status. The genetic map-based appraisals provide support for postulated early divergence of *L. multiflorum* and *L. perenne* and thus confirm the utility of genetic maps in evolutionary studies. In the light of the present data, it is obvious that a refined understanding of evolutionary processes is possible if the precise loci, sequences or chromosome regions underlying differences between species can be identified. Techniques for generating large numbers of genetic markers and the availability of markers from different model species are likely to make genetic mapping more common for wild species. Genetic maps also enable to map QTLs including those responsible for early evolution as well as to compare closely related species. Up to the present genetic maps have been by-products of breeding activities and searches for genes responsible for important characters as well as markers for marker assisted selection. Therefore first interspecific comparisons were done for crops. The goal of

the first maps between maize and teosinte was to locate genes that would be useful in enrichment of genetic resources of the former. Surprisingly, these studies were the foundation for using genetic maps of plants for tracking the genomic changes during plant domestication (Doebley and Stec 1993). The sunflower maps were the breakdown in the understanding of the hybrid origin of *Helianthus* annomalus, *H. deserticola* and *H. paradoxus* as well as of the role of allopolyploidy and hybridisation in speciation (Rieseberg and Carney 1998). The present map of *L. multiflorum* and *L. perenne* has been constructed with the aim to improve our understanding of the process of their evolution. The remaining question is whether *L. multiflorum* and *L. perenne* differ with respect to quantitative traits that might drive their future speciation similarly to that observed during domestication of maize. The high resolution of the constructed map opens the doors to the challenging task of detecting QTLs affecting a variety of discrete traits responsible for some taxonomic characters. The dissection of these traits through QTL mapping would represent a major drawback towards the final clarification of the evolutionary processes in *L. multiflorum* and *L. perenne*.

6.5. CONCLUSIONS

1. A relatively little genetic incompatibilities are found between two botanical taxa *L. multiflorum* and *L. perenne* and no signs of a reproductive barrier are observed. The majority of markers segregate in the Mendelian fashion. The level of distortions is comparable in intra- and interspecific crosses. Selection against transposon insertions provides evidence that intraspecific mechanisms protecting from undesired mobilisation of transposons are well working in the population derived from the cross between *L. multiflorum* and *L. perenne*. All these data fully confirm our previous view that *L. multiflorum* and *L. perenne* can not be regarded as biological species. Therefore, their taxonomic rank should be lowered and the subspecies level seems to be more appropriate.
2. Distorted regions are scattered throughout the whole genome and they are correlated with the presence of transposons.
3. The significant distortions from the expected Mendelian ratios observed for the majority of transposons confirm their role in the early steps of evolution.
4. Application of a wide range of different types of molecular markers is a necessary condition to prevent from marker clustering on genetic maps and furthermore to construct high resolution genetic maps without substantial gaps. The current genetic map consists of 502 markers including 16 isozymes, 132 RAPD markers, 80 ISJs, 6 SSRs, 137 AFLPs, 57 insertion sites of the DNA transposon *Tpo1*, 39 of the retrotransposon *Lolcopia2*, and 33 markers derived from *M. tuberculosis* genes.
5. Genetic maps are powerful tools for studying speciation at the very early stages, prior to reproductive barrier formation.
6. Genetic mapping confirms the utility of bacteria-derived sequences for studies in plants. All polymorphic B-SAP markers are mapped. They exhibit the dominant mode of inheri-

tance; the presence of a band is dominant over its lack. Moreover, these types of markers rarely show segregation distortions.

7. The linkage between katG markers and peroxidase loci supports the idea that katG primers complementary to the *M. tuberculosis* catalase-peroxidase gene amplify plant peroxidases. The linkage between katG markers and the other enzymatic loci proves that these markers are predominantly correlated with genes encoding enzymes.
8. Seedling root fluorescence that is regarded as marker discriminating *L. multiflorum* from *L. perenne* is encoded by two complementary genes located on LG1. In intra- and interspecific populations either both genes or one of them can segregate. This model of inheritance explains well the difficulties in the species separation and stabilisation of the seedling root fluorescence level in cultivars of *L. perenne* so frequently encountered by breeders.

7. ROLE OF QTL_S IN THE EARLY EVOLUTION OF *L. MULTIFLORUM* AND *L. PERENNE*

7.1. INTRODUCTION

The success of a population to become reproductively isolated from the others depends on acquisition of genetic differences (Mayr 1996). Recent mapping studies have revealed genes of large effects associated with reproductive isolation in plants and animals. Examples include a meiotic drive locus (D) responsible for segregation distortions in hybrids between yellow monkeyflowers, *Mimulus guttatus* and *M. nasutus* (Fishman and Willis 2005), 5-11 QTLs that reduce hybrid fitness by 36-90% in *Lycopersicon esculentum* and *L. hirsutum* hybrids (Moyle and Graham 2005), and Hybrid male rescue (*Hmr*) gene causing lethality and hybrid sterility among *D. melanogaster* and its sibling species (Barbash et al. 2003). These data are in agreement with one long-standing view that "major genes" with large allelic effects are playing a central role in speciation (Comes 19-98). Single mutations of a special kind — "macromutations" may be responsible for sudden transformation of one kind of organism into another. Tallies of induced mutants in crops provide sufficient data that few-gene changes are able to create novel phenotypes. To pick just two examples, *P. sativum* mutant with mutated dim sector is characterized by female sterility while brachytic mutant of *H. vulgare* exhibits about 80% of growth reduction in comparison with wild form (K. Polok, unpublished data). Studies of domestication routes in maize have elegantly demonstrated how dramatic morphological effects can result from changes in expression pattern of only a single gene. The teosinte *branched1* (*tb1*) gene is upregulated in maize and causes a repression of organ growth in those tissues in which its messenger RNA accumulates. This is visible as the reduction of axillary branches in maize, short ear shoots and tassel-tipped branches (Doebley and Stec 1993; Doebley 2004).

A counterproposal frequently expressed is that speciation is a continuation of the same microevolutionary processes that are responsible for differences between conspecific populations (Avise 2004). Two populations gradually diverge through the fixation of different alleles at a number of loci and eventually acquire reproductive isolation. Consequently, even closely related species normally differ in many genes. This theory was proposed simultaneously by Dobzhansky and Muller many years ago (Barbash et al. 2003) but recently it has been strongly supported by analyses of reproductive barriers in sibling species of *Drosophila*. Hybrid incompatibilities prove attributable to differences in many loci with relatively small effects (Tao et al. 2003). A survey of allozyme studies, recently replenished with DNA data, has also indicated that a substantial degree of genetic differentiation occurs during the first stages of speciation prior to the completion of intrinsic reproductive barrier. At the very early stages evolutionary divergence often involves discrete differences in quantitative characters

encoded by many genes i.e., quantitative trait loci (QTL). A quantitative trait locus is a gene or group of genes that affects a trait measured on a linear scale. QTLs are identified via statistical procedures and they are assigned to a genetic map based on a linkage between two adjacent markers. QTLs are located to the genome regions using conditional probabilities (Lynch and Walsh 1998). Because each gene has small effect on phenotype, very often below resolution power of statistical methods, moreover each gene interacts with a great number of other genes and environment, at first glance; variation at quantitative loci is invisible and seems not accessible to selection. Under Lauter and Doebley (2002) model, variation at quantitative loci is called cryptic variation while discrete traits - threshold traits. This means that a population would rise above the certain threshold, for example detectable by statistical methods, only if allelic variation is gathered at multiple QTLs. Selection would then favour new multigenic combinations that create a discrete shift in morphology. In the end, these accumulated differences may lead to species differentiation.

Despite the point has been made many times that evolutionary divergence often involves polygenic characters, experimental support has been very weak due to a lack of suitable tools. In general, the approach based on experimental crosses and analysing quantitative characters in segregating progeny has been limited to model species such as *Drosophila*. The dissection of complex phenotypes has become possible only alongside with advances in genetic mapping. Identification of the number and genomic positions of quantitative trait loci (QTLs) underlying morphological or other differences between species of interest opens the door to routine characterisation of the genetics of speciation. Results from several QTLs studies confirm that interspecific differentiation is under polygenic control and responsible genes are widespread across the whole genome. Examples involve the morphological traits distinguishing maize and teosinte (Doebley 2004); wild emmer wheat, *Triticum dicoccoides* from modern tetraploid wheat, *T. durum* (Peng et al. 2003), foxtail millet, *Setaria italica* from green millet, *S. viridis* (Doust et al. 2005), oak species, *Quercus petraea* and *Q. robur* (Saintagne et al. 2004) and many others (Orr 2001).

At the very early stages of evolution, populations that differ only in quantitative characters are often completely interfertile when artificially mated although natural hybrids are rarely observed. Two species of monkeyflowers, *Mimulus lewisii* and *M. cardinalis* grow and flower together, yet they are reproductively isolated owing to different preferences of pollinators. The former with pink petals, contrasting yellow nectar guides and wide corolla is pollinated by bumble-bees whereas the latter with red petals and a narrow, tubular corolla is pollinated by hummingbirds. Regardless of these differences, no postzygotic reproductive barrier exists between them and if monkeyflowers are crossed experimentally, interspecific hybrids are fully fertile. Subsequent genetic analyses have also revealed that several QTLs are responsible for the floral traits and presumably pollinator preferences (Bradshaw et al. 1998). Obviously, despite striking morphological differences, such populations can not be regarded as biological taxa. Maize and its closest relatives, the teosintes exhibit such extreme differences in morphologies that taxonomists used to classify the teosintes closer to rice than to maize. Notwithstanding such views, the genomes of maize and teosinte are very similar; both taxa share the identical chromosome morphologies and their hybrids exhibit completely normal meiosis and full fertility. Because teosinte is a wild plant while maize is known only in cultivation it has been proposed that maize

is simple a domesticated form of teosinte. At present both forms are classified within the same biological species with a rank of subspecies i.e., *Z. mays* ssp. *mays* for maize and *Z. mays* ssp. *mexicana* or *Z. mays* ssp. *parviglumis* for teosinte. The key morphological traits differentiating maize and teosinte are the result of human selection during domestication and they are controlled by several major QTLs mapped within five chromosomal regions (Doebley and Stec 1993; Doebley 2004). Much excitement, therefore, attends QTL analyses as a means to answer various basic questions. How are reproductive barriers formed? What genes are responsible for producing adaptations and organismal diversity? Variation at QTLs does unequivocally not testify for speciation, nevertheless, QTL mapping might indicate the direction towards which populations will have been proceeding.

With regard to *L. multiflorum* and *L. perenne*, genetic diversity and mapping studies have likewise suggested that these two botanical taxa are at the very early stages of divergence. Similarly to maize and teosinte, they have very similar genomes (Chapter 4, 6); the same karyotypes and their hybrids are fully fertile. Genetic mapping provides evidences that no intrinsic reproductive barrier exists between them. Therefore they can not be regarded as biological species under the biological species concept (BSC). Nevertheless, a conclusion emerging from insertional polymorphism and transposon mapping is that their diversification might have just begun. It is plausible therefore, that *L. multiflorum* and *L. perenne* evolution may be envisioned as a discrete shift from one niche to a new, essentially unoccupied niche. For instance, they can adapt to dryer environments (*L. multiflorum*, Chapter 4) as well as they can invade niches with much reduced competitions such as cultivated fields (Chapter 3-6). Under Lauter and Doubley “threshold hypothesis” (2002), one can easily imagine that *L. multiflorum* and *L. perenne* do not differ statistically in the majority of morphological characters (Chapter 3) because differences are below a certain threshold. However, some cryptic variation presumably exists in populations of both species. Consider such characters as floret number or flag leaf width, which have been the subject of several taxonomic and genetic studies including the present work. Despite overlapping ranges of variation some discrete shifts do exist, for example somehow narrow range of floret numbers in *L. perenne* or discrete shift of the flag leaf means. If these differences are determined genetically, it should be possible to identify QTLs responsible for them. These QTLs can be examples of “speciation genes” responsible for future species diversification. Determining the nature of genetic events involved in the proposed evolution of *L. multiflorum* and *L. perenne* is fundamental for understanding the changes during early steps of their speciation. Although quantitative phenotypic differences between *L. multiflorum* and *L. perenne* as a basis for dichotomous taxonomic keys have received extensive attention from botanists, surprisingly little investigation has been devoted to the genetics of quantitative changes surrounding the diversification of these taxa. In *Lolium*, similarly to other crops, QTL analyses have resulted from breeders' needs for improved and highly adapted germplasm and therefore have been focused on structural characteristics important for herbage yield and predictions of the response to grazing. To date there have been relatively few reports analysing QTLs in *Lolium* and no reports taking into account the evolutionary point of view.

Therefore the goal of this part of research was to estimate the number of genes (QTLs) controlling quantitative characters, the magnitude of their effects and the genomic organization. Because *L. multiflorum* and *L. perenne* have been evolved only recently, it is likely to

identify changes that are occurring during the process of diversification and that might lead to separation into different species in the future. Moreover, growing in semi-natural habitats and being important fodder and turf crops and thus being under strong selection during breeding, *L. multiflorum* and *L. perenne* can be a good model allowing understanding how species respond evolutionarily to growing pressure from humans. It has to be pointed out, however, that any generality of speciation scenarios is difficult. This is in part because many of the genetic events associated with speciation often occur at the population level as well without producing new species (Avise 2004).

7.2. MATERIAL AND METHODS

7.2.1. Comparison of quantitative characters in intra- and interspecific crosses

To estimate the role of quantitative characters in the early evolution of *L. multiflorum* and *L. perenne*, these traits were analysed in four F_2 populations involved both inter- and intraspecific crosses. Two F_2 populations, namely BR3 x NZ15 and HU5 x BO2 represented interspecific level, whereas populations VA7 x AS17 and KY20 x BB6 intraspecific level, *L. multiflorum* and *L. perenne*, respectively. The detailed description of these populations, plant development and field experiments are given in Annex 13.1. Shortly, a randomized complete block design with 3 blocks was used to analyze all populations. Each block consisted of three replications and each replication consisted of three ramets as replicates of each genotype. Hence, in each block a single genotype was represented by nine ramets. Quantitative characters were analysed during two seasons and during two crops in each season. Where possible each plant was scored for 21 morphological characters. The following traits were measured, basal leaf length, basal leaf width, green weight and dry weight of vegetative leaves, height at ear emergence, spike length, spikelet length, spikelet number, floret number, flag leaf length and flag leaf width as well as green and dry weight of generative tillers. Basal and flag leaf areas were recalculated from length and width. In addition such characters as a growth type, leaf colour, resistance to crown rust (*Puccinia coronata* f. sp. *lolii*) and presence of awns were estimated during each crop by visual investigations. For growth types, the number 1 indicated prostrate growth type while 5 erect, for leaf colour the number 1 was assigned to very light green and 5 to very dark green, for rust the number 1 means resistant without any signs of a disease and 9 completely susceptible with necrotic areas on the whole leaves. The presence of awns was described as 1, the absence as 0, and 0.5 was assigned to all intermediate types. Similarly, plant survival in the field was estimated in the second year of cultivation by a visual assessment score (as percentage) of plant recovery. Days to ear emergence were counted from the beginning of spring rush.

Data were analysed using STATISTICA 7.1 software. Skewness was calculated for each trait to determine the extent to which it deviated from normality. For each cross combinations mean values of all analysed traits were estimated for parents, F_1 hybrids and F_2 individuals. Moreover, mean, minimum and maximum values were calculated for each F_2 population. Means of F_2 individuals, parents and F_2 were calculated taking into account two

crops in two years of cultivation. Thus, factorial analysis of variance within general linear model (GLM) was used to check for genotype, environment (year or crop) and interaction genotype x environment effect. Significant differences between genotypes were analysed by the LSD test.

For each hybrid the level of heterosis was estimated as an increase over the better parent. Heterosis was defined here, according to of G.H. Shull (Shull 1952), as the increase in size, vigour, yield, disease resistance and other quantitative traits in F_1 hybrids in comparison with the better parent. This definition points at the advantage of the F_1 hybrid over each parent. Therefore, the level of heterosis with regard to a given trait should be estimated as the increase over this parent, which exhibits the higher value of a trait. In the light of the Shull's definition, it is misunderstanding to measure heterosis in relation to mid-parent value although some authors tend to do it (Polok 1996).

7.2.2. QTL mapping

Quantitative trait loci were mapped in the interspecific F_2 population derived from a cross between *L. multiflorum* and *L. perenne*, BR3 x NZ15, for which the genetic map was previously constructed (Chapter 6). A set of 502 genetic markers from this map was combined with a dataset from quantitative traits. QTL mapping was done first with MAPL98 (Ukai 2004) using interval mapping by maximum likelihood. The principle behind this method was to test for the presence of a QTL at many positions between two mapped markers. The likehood of the observed distributions of a QTL effect was computed and the map position of a QTL was determined as the maximum likelihood from the distribution of likelihood values (LOD scores). The 1 cM intervals and a minimum LOD threshold of 3.0 were selected for significance of location of QTLs. The genetic map of *L. multiflorum* and *L. perenne* had high resolution, the density of markers was high and they were rather equally distributed on each linkage group with mean distance of around 2 cM thereby, the same critical LOD score could be employed for all linkage groups. If evidence for more than one QTL peak was found on the same linkage group, the trait was analysed by composite interval mapping with multiple regression using Windows QTL Cartographer 2.5 (Wang et al. 2007). Similarly, to simple interval mapping, this method tests hypothesis of a QTL presence in an interval between two adjacent markers, however, at the same time it tests the effects of segregating QTLs elsewhere in the genome. Additional criterion was employed for QTLs in close proximity. They were accepted only when score dip between the QTLs peaks was bigger than 2 LODs.

The magnitude of QTL effect was estimated as the percentage of F_2 phenotypic variance explained (PVE). An arbitrary criterion of minimum 25% PVE was used to define a major QTL (Bradshaw et al. 1998). The additive and dominance effects of each QTLs were tested. The sign of the effects indicated the direction of change in the phenotype. A positive sign indicated an increase and negative sign a decrease in the trait value caused by a given QTL.

7.3. RESULTS

7.3.1. Heterosis in intra- and interspecific crosses

All hybrids, either from interspecific crosses or intraspecific ones were fully fertile and no signs of sterility were noticed. All hybrids were similar or better than parents with regard to all analysed quantitative characters (Table 7.1). Hybrids had phenotypes of better parents for 50-60% of characters, from 10% to almost 40% characters were intermediate and for about 25-30% heterosis occurred (Figure 7.1). In no case, hybrids were worse than parents. For example, the interspecific hybrid derived from the cross between *L. multiflorum* and *L. perenne*, BR3 x NZ15, was much the same *L. multiflorum* parent, which exhibited higher values of analysed traits. However, it has slightly darker leaves that were rather similar to *L. perenne* and it has somehow intermediate awns. It also survived winter in a much better condition than parents. The F_1 and parents of another interspecific combination did not differ statistically for the majority of characters although the tendency of the hybrid to be similar to the better parent or intermediate could be realized. The hybrid of *L. multiflorum* was characterized by extremely long spike and big basal leaves whereas the highest weight of tillers was typical of the *L. perenne* hybrid (Table 7.1).

Heterosis was observed in all intraspecific cross combinations and in an interspecific one. Only in the cross, HU5 x BO2, the F_1 hybrid did not exceed significantly parents. Frequencies and the magnitudes of heterosis were similar in inter- and intraspecific crosses. In total seven traits had higher values in the F_1 from the interspecific cross, BR3 x NZ15 (33%) and five in hybrids from intraspecific crosses (24 and 25%). All these three F_1 hybrids exhibited heterosis for spike length and green weight of tillers and two of them for dry weight of tillers (Figure 7.2). Moreover, the interspecific hybrid, BR3 x NZ15 exhibited heterosis for spikelet and flag leaf traits while the *L. multiflorum* hybrid for basal leaf traits (Table 7.1). The unexpected result was obtained for the *L. perenne* hybrid, in which heterosis was observed for one of the most important characters differentiating *L. multiflorum* from *L. perenne* i.e., number of florets per a spikelet. The hybrid had 35% more flowers than the better parent (Figure 7.2), all the more, numerically the number of flowers was even higher than in the *L. multiflorum* hybrid. It is worthy to notice that this trait was intermediate or similar to better parents in the remaining hybrids (Figure 7.2).

When we turn instead to analysed traits, among 21 of them, 12 exhibited heterosis in at least one F_1 hybrid. Heterosis for spike characters, weight of tillers and basal leaf traits was observed the most frequently. The magnitude of increase depended on a trait. The most extreme values were observed for weight of tillers. Hybrids had higher green weight of 140-160% and dry weight of more than 200% in comparison with better parents. Surprisingly, green and dry weight of vegetative parts in any F_1 hybrid did not exceed the values of parents.

Table 7.1. Mean values, minimum and maximum for 21 quantitative traits in four cross combinations of *L. multiflorum* and *L. perenne*

Trait	Value	Cross combination			
		Interspecific		Intraspecific	
		BR3 x NZ15 (*L. multiflorum* x *L. perenne*)	HU5 x BO2 (*L. perenne* x *L. multiflorum*)	VA7 x AS17 (*L. multiflorum*)	KY20 x BB6 (*L. perenne*)
Basal leaf length [cm]	P_1 (mean)	21.19[a]	23.75	24.07[a]	15.97[a]
	P_2 (mean)	16.92[b]	26.60	27.70[a]	22.93[b]
	F_1 (mean)	20.04[a]	26.78	**35.22[b]**	18.60[ab]
	F_2 (mean)	23.22	24.54	24.09	24.36
	F_2 (minimum)	12.59	13.13	10.48	12.05
	F_2 (maximum)	29.27	32.75	34.47	36.58
Basal leaf width [cm]	P_1 (mean)	0.24[a]	0.23[a]	0.42[a]	0.12[a]
	P_2 (mean)	0.25[ab]	0.38[b]	0.39[a]	0.42[b]
	F_1 (mean)	0.28[b]	0.28[ab]	**0.58[b]**	0.26[ab]
	F_2 (mean)	0.32	0.24	0.40	0.28
	F_2 (minimum)	0.20	0.12	0.17	0.17
	F_2 (maximum)	0.50	0.37	0.65	0.43
Basal leaf area [cm^2]	P_1 (mean)	5.32[ab]	5.83	10.43[a]	1.85[a]
	P_2 (mean)	4.29[b]	11.09	11.14[a]	9.59[b]
	F_1 (mean)	5.79[a]	7.28	**21.49[b]**	4.92[ab]
	F_2 (mean)	7.90	6.52	10.03	7.40
	F_2 (minimum)	2.77	1.85	1.93	2.50
	F_2 (maximum)	14.29	12.70	19.59	15.77
Green weight [g]	P_1 (mean)	85.86[a]	76.02	44.93	31.75[a]
	P_2 (mean)	37.10[b]	87.33	55.93	70.25[b]
	F_1 (mean)	91.94[a]	95.18	60.87	64.10[b]
	F_2 (mean)	123.48	51.39	34.81	59.02
	F_2 (minimum)	7.40	2.17	1.30	12.12
	F_2 (maximum)	271.07	167.12	166.67	110.30
Dry weight [g]	P_1 (mean)	19.17[a]	19.00	17.47	11.58[a]
	P_2 (mean)	9.29[b]	25.53	23.80	20.38[b]
	F_1 (mean)	20.67[a]	28.52	23.57	18.38[b]
	F_2 (mean)	26.24	11.80	6.73	15.45
	F_2 (minimum)	1.78	0.97	0.23	2.92
	F_2 (maximum)	63.77	29.93	48.07	32.27
Growth type 1=prostrate 5=erect	P_1 (mean)	2.46[a]	3.32[a]	4.11	2.22[a]
	P_2 (mean)	1.72[b]	4.30[b]	3.20	1.58[b]
	F_1 (mean)	2.67[a]	3.23[a]	4.00	2.33[a]
	F_2 (mean)	2.77	2.89	3.67	2.59
	F_2 (minimum)	1.44	1.33	1.00	2.44
	F_2 (maximum)	4.22	4.67	5.00	4.67
Leaf colour 1=very light green 5=very dark green	P_1 (mean)	2.73[a]	3.22[a]	2.00	3.44[a]
	P_2 (mean)	3.57[b]	2.53[b]	1.33	4.25[b]
	F_1 (mean)	3.49[b]	2.99[a]	1.53	4.22[b]
	F_2 (mean)	3.26	3.20	2.49	3.32
	F_2 (minimum)	2.06	1.50	1.01	2.44
	F_2 (maximum)	4.26	4.33	4.97	4.67
Crown rust resistance 1=resistant 9=susceptible	P_1 (mean)	3.72[a]	3.10[a]	4.51	2.33
	P_2 (mean)	2.55[b]	3.94[b]	6.32	3.42
	F_1 (mean)	3.36[a]	2.76[a]	5.15	3.33
	F_2 (mean)	3.01	2.60	3.98	2.24
	F_2 (minimum)	1.85	1.25	2.00	1.64
	F_2 (maximum)	4.96	5.50	6.53	3.33

[abc]different letters mean significant differences at P=0.05 for LSD test, bold – heterosis was observed in F_1 for a given trait

Table 7.1. Mean values, minimum and maximum for 21 quantitative traits in four cross combinations of *L. multiflorum* and *L. perenne*, continued

Trait	Value	Cross combination			
		Interspecific		Intraspecific	
		BR3 x NZ15 (*L. multiflorum* x *L. perenne*)	HU5 x BO2 (*L. perenne* x *L. multiflorum*)	VA7 x AS17 (*L. multiflorum*)	KY20 x BB6 (*L. perenne*)
Height at ear emergence [cm]	P_1 (mean)	69.56[a]	67.67	60.36	51.50[a]
	P_2 (mean)	47.00[b]	69.17	58.25	38.33[b]
	F_1 (mean)	61.67[a]	64.00	62.16	60.50[a]
	F_2 (mean)	66.81	62.61	48.98	62.48
	F_2 (minimum)	46.67	34.50	25.03	27.00
	F_2 (maximum)	91.22	87.67	86.04	80.00
Spike length [cm]	P_1 (mean)	18.57[a]	18.97[a]	15.77[a]	12.15[a]
	P_2 (mean)	14.72[b]	20.91[b]	16.93[a]	17.53[b]
	F_1 (mean)	**22.99[c]**	21.24[b]	**27.60[b]**	**19.82[c]**
	F_2 (mean)	20.16	18.58	16.50	19.04
	F_2 (minimum)	12.00	12.02	7.43	7.20
	F_2 (maximum)	27.47	24.74	27.33	24.08
Spikelet length [mm]	P_1 (mean)	17.29[a]	13.15[a]	14.10	14.83
	P_2 (mean)	14.00[b]	17.06[b]	15.90	11.97
	F_1 (mean)	18.73[a]	16.76[b]	14.57	14.52
	F_2 (mean)	16.84	15.03	12.36	15.56
	F_2 (minimum)	11.78	10.85	6.00	10.98
	F_2 (maximum)	22.18	19.12	18.10	19.57
Spikelet number [n]	P_1 (mean)	17.16[a]	17.30	15.90	10.00[a]
	P_2 (mean)	15.37[b]	18.79	14.00	18.25[b]
	F_1 (mean)	**19.59[c]**	17.42	19.67	16.18[b]
	F_2 (mean)	17.76	15.88	17.50	15.74
	F_2 (minimum)	9.70	11.65	6.67	6.10
	F_2 (maximum)	29.00	35.38	33.00	18.87
Floret number [n]	P_1 (mean)	9.76[a]	8.06[a]	10.23[a]	7.50[a]
	P_2 (mean)	8.02[b]	10.29[b]	9.10[b]	5.90a
	F_1 (mean)	10.16[a]	9.29[b]	9.57[ab]	**10.12[b]**
	F_2 (mean)	9.66	8.69	7.37	9.04
	F_2 (minimum)	5.56	5.03	3.33	5.50
	F_2 (maximum)	12.14	13.60	11.43	11.93
Flag leaf length [cm]	P_1 (mean)	14.81[a]	18.09	9.53[a]	11.88[a]
	P_2 (mean)	11.29[b]	17.90	16.40[b]	14.62[b]
	F_1 (mean)	**18.58[c]**	17.72	16.17[b]	12.70[ab]
	F_2 (mean)	13.70	15.78	12.83	16.72
	F_2 (minimum)	8.00	7.73	3.97	9.35
	F_2 (maximum)	19.64	21.68	23.37	20.73
Flag leaf width [cm]	P_1 (mean)	0.41[a]	0.31[a]	0.33[a]	0.20[a]
	P_2 (mean)	0.31[b]	0.40[b]	0.57[b]	0.35[b]
	F_1 (mean)	**0.51[c]**	0.40[b]	0.52[b]	0.33[b]
	F_2 (mean)	0.41	0.29	0.42	0.28
	F_2 (minimum)	0.30	0.17	0.20	0.23
	F_2 (maximum)	0.85	0.49	0.70	0.38
Flag leaf area [cm^2]	P_1 (mean)	6.14[a]	5.58	3.19[a]	2.38[a]
	P_2 (mean)	3.47[b]	7.14	9.46[b]	5.39[b]
	F_1 (mean)	**9.48[c]**	7.29	8.42[b]	4.18[b]
	F_2 (mean)	5.81	4.74	5.63	4.82
	F_2 (minimum)	2.40	1.42	1.10	2.21
	F_2 (maximum)	12.55	10.34	14.29	7.34

[abc]different letters mean significant differences at P=0.05 for LSD test, bold – heterosis was observed in F_1 for a given trait

Table 7.1. Mean values, minimum and maximum for 21 quantitative traits in four cross combinations of *L. multiflorum* and *L. perenne*, continued

Trait	Value	Cross combination			
		Interspecific		Intraspecific	
		BR3 x NZ15 (*L. multiflorum* x *L. perenne*)	HU5 x BO2 (*L. perenne* x *L. multiflorum*)	VA7 x AS17 (*L. multiflorum*)	KY20 x BB6 (*L. perenne*)
Green weight of tillers [g]	P_1 (mean)	58.49[a]	208.59[a]	13.50[a]	40.02[a]
	P_2 (mean)	55.77[a]	81.85[b]	15.47[a]	155.17[b]
	F_1 (mean)	**157.01[b]**	115.83[ab]	**30.83[b]**	**382.95[c]**
	F_2 (mean)	155.95	181.24	17.78	199.98
	F_2 (minimum)	6.51	7.45	1.10	10.67
	F_2 (maximum)	378.6	442.25	76.73	359.53
Dry weight of tillers [g]	P_1 (mean)	13.28[a]	56.51[a]	2.67	25.78[a]
	P_2 (mean)	14.09[a]	20.36[b]	2.60	36.97[a]
	F_1 (mean)	**44.62[b]**	29.11[b]	6.10	**130.67[b]**
	F_2 (mean)	41.04	46.42	4.22	50.63
	F_2 (minimum)	1.80	2.35	0.20	1.23
	F_2 (maximum)	96.30	110.28	16.02	85.97
Winter survival [%]	P_1 (mean)	40.56	88.33	11.00	38.33
	P_2 (mean)	51.11	30.00	5.00	58.33
	F_1 (mean)	65.56	57.83	10.33	**81.67**
	F_2 (mean)	53.19	73.66	11.10	80.91
	F_2 (minimum)	14.56	18.33	0.00	19.33
	F_2 (maximum)	88.33	96.67	21.33	96.67
Days from spring rush to ear emergence [n]	P_1 (mean)	47.56[a]	51.33	Not analysed	45.00[a]
	P_2 (mean)	45.89[ab]	47.33		57.00[b]
	F_1 (mean)	46.89[a]	50.33		46.83[a]
	F_2 (mean)	48.20	47.27		47.29
	F_2 (minimum)	41.33	43.00		44.00
	F_2 (maximum)	62.00	54.67		56.00
Presence of awns 1=presence 0=absence 0.5=intermediate	P_1 (mean)	0.89	0.00	1.00	0.00
	P_2 (mean)	0.00	1.00	0.91	0.11
	F_1 (mean)	0.72	1.00	0.95	0.05
	F_2 (mean)	0.40	0.60	0.87	0.00
	F_2 (minimum)	0.00	0.00	0.25	0.00
	F_2 (maximum)	1.00	1.00	1.00	0.08

[abc]different letters mean significant differences at P=0.05 for LSD test, bold – heterosis was observed in F_1 for a given trait

There was no clear connection between the level of heterosis and genetic diversity of parents based on RAPD, ISJ and enzymatic data. When interspecific cross combinations were compared, it was more or less obvious that heterosis was typical of crosses between more diverse parents. Genetic similarity between BR3 and NZ15 was quite low and reached 0.588 and these parents produced the heterotic hybrid for seven characters. On opposite, genetic similarity between HU5 and BO2 was 0.754 and no heterosis was observed in F_1. Unfortunately, this rule was not true for intraspecific crosses. Parents of both *L. multiflorum* and *L. perenne* hybrids were the most similar with I value equal to 0.870 and 0.842, respectively, yet heterosis was observed quite frequently (25% of traits). Even more surprising was extremely high green and dry weight of tillers in the *L. perenne* hybrid that could not be predicted based on rather poor performance of the parents.

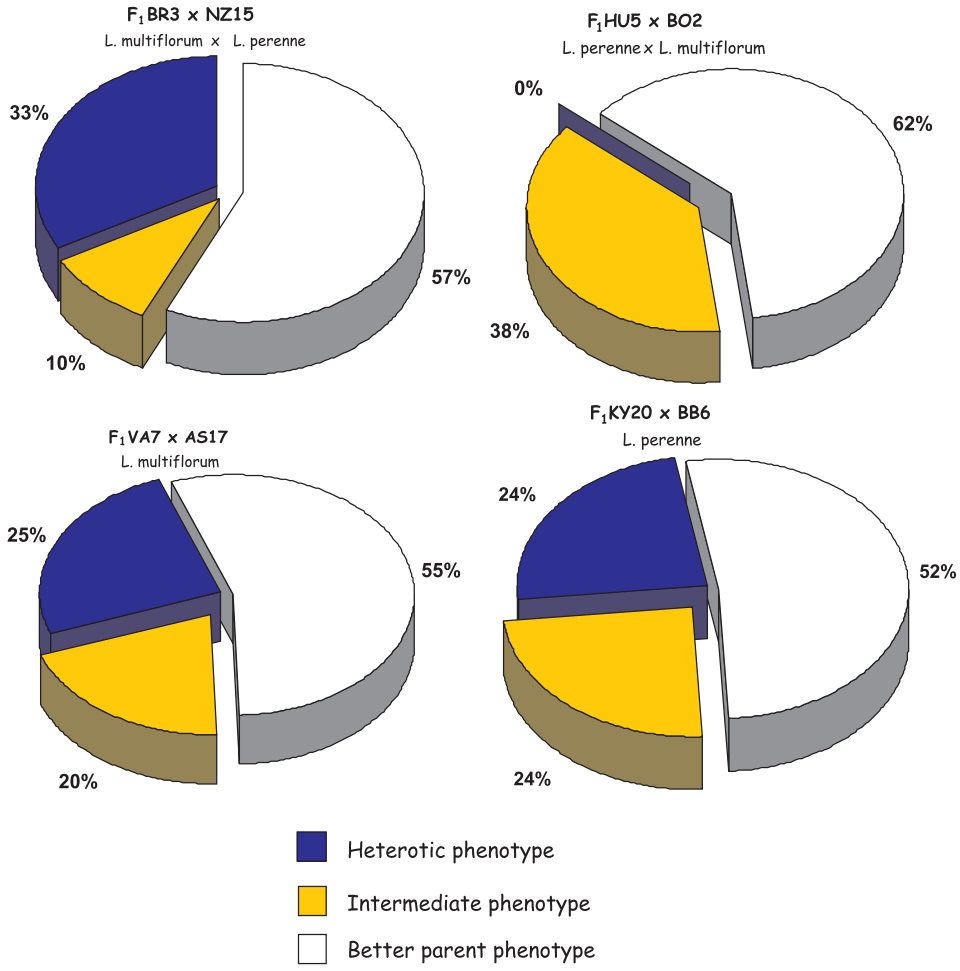


Figure 7.1. Phenotypes of F_1 hybrids of intra– and interspecific crosses of *L. multiflorum* and *L. perenne*

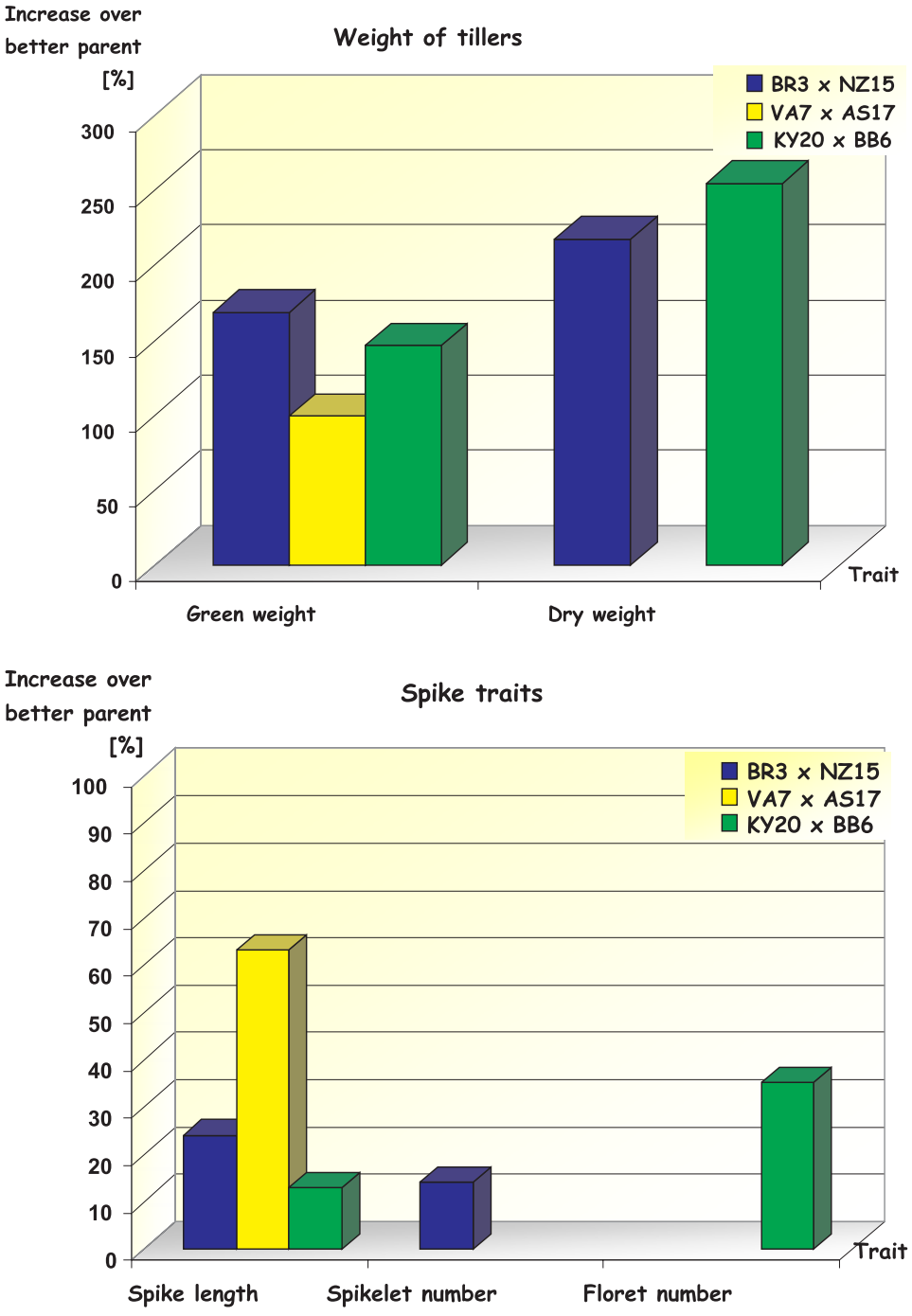


Figure 7.2. Heterosis in F_1 hybrids of intra- and interspecific crosses of *L. multiflorum* and *L. perenne*

7.3.2. Variation of quantitative traits in F_2 populations from intra- and interspecific crosses

All analysed F_2 populations were characterized by great variation in quantitative characters, far ahead the values typical of parents (Figure 7.3). It was emphasized by numerous transgressive forms both with minimal and maximal expression of traits, which were present in all populations; irrespectively a population was derived from an intra- or interspecific cross (Table 7.1). Genotypes with minimum expression of quantitative characters had values of analysed traits on average 47% lower than worse parents while those with maximum expression exceeded better parents of about 76%. In a certain degree the magnitude of increase or decrease depended on a kind of character and F_2 population. Unsurprisingly, the smallest decrease and increase in values of traits were observed for such morphological characters as growth type, leaf colour and crown rust resistance. The extreme genotypes had about 35% more prostrate growth, lighter colour and were more susceptible to crown rust than parents. In principle, the worst genotypes were characterized by very low values of both vegetative traits including survival during winter and generative characters. They were worse than parents on average of 65 and 43%, respectively. The most typical feature of the worst genotypes was the extremely low weight of vegetative parts and tillers.

Figure 7.3. Variation of quantitative characters in the F_2 population derived from the cross between *L. multiflorum* and *L. perenne*, BR3 x NZ15

By contrary, the best transgressants being almost erect, rather dark green and a little more resistant to crown rust overcame parents by roughly 42%. With regard to vegetative and generative characters they exceeded parents of about 70 and 91%. Again, the extreme increases were found for weight of vegetative parts and tillers. For example, green weight could be as much as threefold higher in F_2 genotypes in comparison with parents. And even higher increase, reaching about 500% was observed for green weight of tillers. Besides high weight, the best genotypes had bigger basal leaves, longer spikes and a great number of spikelets in addition to better winter survival. It should be also noted that a given transgressive genotype expressed higher values for the majority of analysed traits at once. This was also a true for the worst genotypes. They performed very poorly for majority of characters.

Whether a population was derived from an interspecific cross or intraspecific one, this did not influence the level of transgression. In contrast, its magnitude seemed to be related rather with parental genotypes. In interspecific crosses both the highest increase and the highest decrease in comparison with parents were observed. This was the F_2 population derived from the cross BR3 x NZ15 (*L. multiflorum* x *L. perenne*), in which the best transgressive forms were twice as good as the better parent (average 128% for all characters). This value was even higher if vegetative and generative characters were considered separately, on average 138% and 146%, respectively (Figure 7.4). A unique feature of this population was that the worst genotypes differed from the parents of as little as 20-30% (Figure 7.5). This was among the lowest values in comparison with the other populations. The opposite relationship was observed in another interspecific cross, HU5 x BO2 (*L. multiflorum* x *L. perenne*). The relatively low increase over the better parent and the great decrease below the worse parent were typical features of it. These differences in behaviour of both populations were likely resulted from performance of parents. The parents of the former population had generally lower values than those of the latter.

If we turn instead to intraspecific crosses, the similar relationships were observed. In the *L. multiflorum* population the variation was shifted towards the lower values. This was emphasized by extremely high decreases of all characters in transgressive forms, and only modest increases of the best genotypes over the better parent for all but generative traits. In that case, the unexpectedly high increase resulted primarily from very low dry weight of parents. The *L. perenne* population could be described by the lowest differences between maximum and minimum. The range of variation of floret number, comparable to that of interspecific crosses, was the most interesting feature of this population. It is worthy to notice that the maximum number of florets in *L. multiflorum* and *L. perenne* populations were almost identical.

Distributions of half quantitative characters analysed in all populations were nearly normal that suggested many genes with presumably additive effects and genotype x environment interactions were responsible for the performance of these traits. Significant departures from normality were observed for about 45% of characters and the number of deviated histograms did not depend on whether a population originated from interspecific or intraspecific crosses (Table 7.2). The asymmetric distribution, suggesting non-additive gene interactions was the most frequent deviation, although some others were also noticed. A striking example was coming from rust resistance, for which five qualitative classes were noticed in the *L. multiflorum* population. Somehow qualitative variation involving

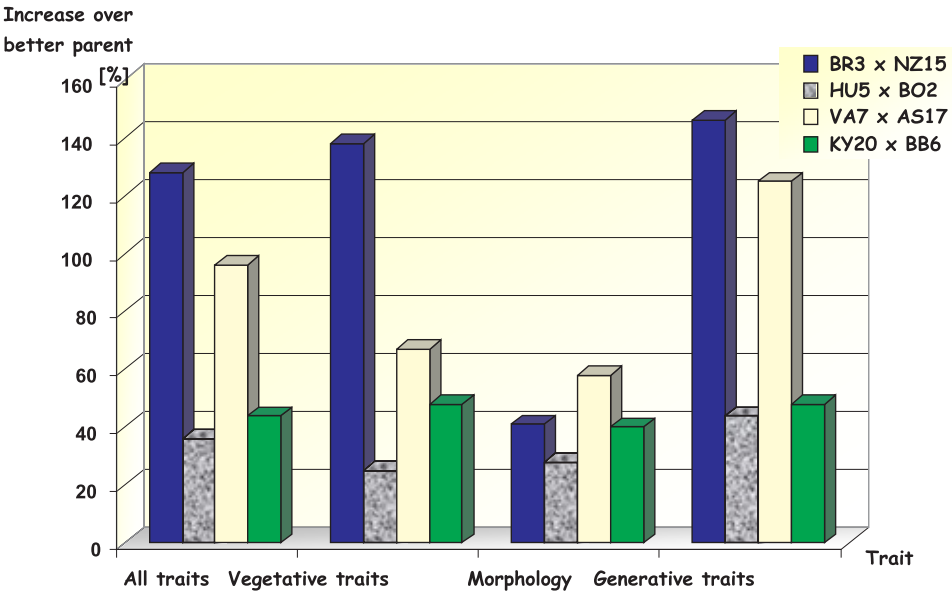


Figure 7.4. Mean increase of trait values in transgressants from the four F_2 populations of *L. multiflorum* and *L. perenne*

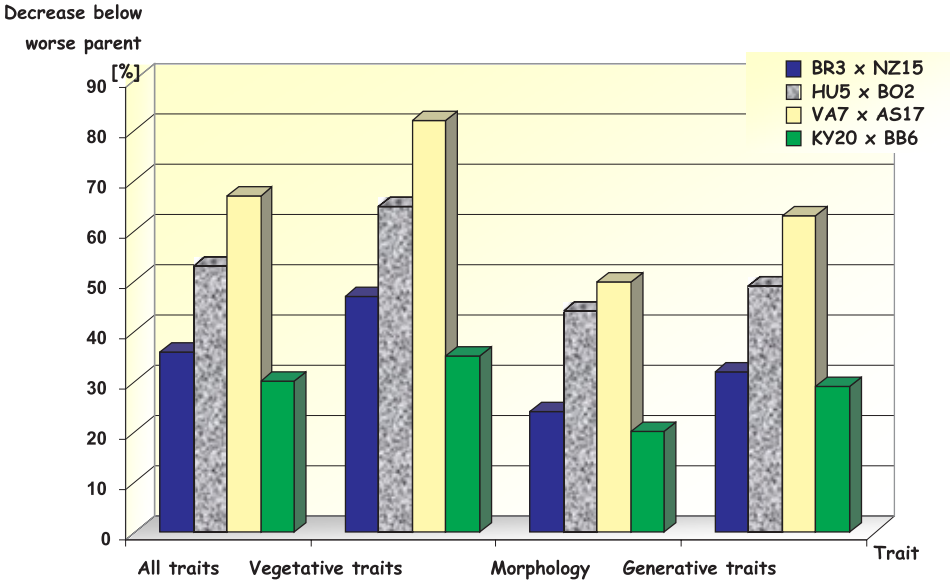


Figure 7.5. Mean decrease of trait values in transgressants from the four F_2 populations of *L. multiflorum* and *L. perenne*

four distinct classes with several minor classes in between was also pronounced in the *L. perenne* population. This may indicate that Mendelian factors were responsible for rust resistance in these cross combinations. However, a caution should be made that bi-

nomial distribution typical of Mendelian factors or major genes was not observed for any trait in any population. Rust resistance was also unusual in that its distribution deviated from normality in both interspecific populations but asymmetric distributions would have indicated many genes with small effect. Summarizing, the highest number of deviated histograms was found in the *L. perenne* population (65%) following by the interspecific one, HU5 x BO6 (50%). About 30% of characters demonstrated departure from normality in the *L. multiflorum* and interspecific, BR3 x NZ15 populations.

Table 7.2. Escape from normality for quantitative traits in F_2 populations of *L. multiflorum* and *L. perenne*

Trait		Skewness			
		Interspecific		Intraspecific	
		BR3 x NZ15 (*L. multiflorum* x *L. perenne*)	HU5 x BO2 (*L. perenne* x *L. multiflorum*)	VA7 x AS17 (*L. multiflorum*)	KY20 x BB6 (*L. perenne*)
Basal leaf length		-0.609*	-0.585*	-0.220	0.023
Basal leaf width		0.439	0.228	-0.940	0.886*
Basal leaf area		0.074	0.044	0.064	1.248*
Green weight		0.117	0.734*	1.667*	0.065
Dry weight		0.269	0.706*	2.528*	0.582*
Growth type		0.041	0.042	-0.108	0.529
Leaf colour		-0.373	-0.454	0.476	0.620*
Crown rust resistance		0.915*	0950*	0.074	**0.372**
Height et ear emergence		0.072	-0.733*	-0.014	-2.056*
Spike length		0.284	0.158	-0.083	-1.748*
Spikelet length		-0.099	-0.233	-0.389	-0.049
Spikelet number		1.366*	3.011*	0.132	-2.452*
Floret number		-0.507	0.417	-0.220	-0.394
Flag leaf length		0.184	-0.059	0.424	-0.707*
Flag leaf width		2.313*	0.656*	0.576*	0.640*
Flag leaf area		0.990*	0.831*	0.972*	0.238
Green weight of tillers		0.287	-0.035	1.850*	-0.482
Dry weight of tillers		0.342	-0.004	1.770*	-0.664*
Winter survival		-0.183	-1.247*	-	-2.056*
Days to ear emergence		1.273*	-0.733*	-	2.292*
Escaped traits	**N**	**6**	**10**	**6**	**13**
	%	**30**	**50**	**33**	**65**

Escape from normality was estimated by skewness and visual inspection of histograms
*Significant different from zero – the distribution was asymmetric, Bold – escape from normal distribution, but not asymmetric

Significant genotype x environment interaction was observed for majority of traits in all crosses as estimated by F value for interactions in factorial ANOVA. This interaction resulted from much lower values for all characters during the second crop in each year. Nearly for each genotype the trait values during the second crop were on average twofold lower and in a case of weight even three-fourfold lower.

7.3.3. Quantitative trait inheritance in *L. multiflorum* and *L. perenne*

Number of QTLs responsible for quantitative characters

The genetic basis of the great quantitative variation observed in the *L. multiflorum* x *L. perenne* F_2 population, BR3 x NZ15 was confirmed by the identification of as many as 145 QTLs (Table 7.3). They were responsible for 21 quantitative traits and on average each trait was encoded by seven QTLs. It was not surprising that the majority of QTLs (65%) were related with different generative characters because they constituted 57% of all analysed traits. The number of QTLs varied considerably between traits from three for basal leaves, weigh of vegetative parts, height, spike and spikelet length up to 16 for flag leaf length. There was a convincing pattern that the greatest numbers of QTLs were detected for traits not selected during breeding. Examples included flag leaf width and spikelet number encoding by 16 and 14 QTLs, respectively. In addition, 14 QTLs were identified for crown rust resistance. However, owing to the visual estimation of resistance, this number may be overestimated so that these QTLs rather concerning the overall disease resistance than crown rust resistance itself. In contrast, economically important traits such as green and dry weight of vegetative parts were encoded by only three QTLs.

Table 7.3. Description of QTLs responsible for 21 quantitative traits in *L. multiflorum* and *L. perenne*

Trait	Number of QTLs	Phenotypic variance explained [%]		QTL size [cM]		Maximum LOD value	
		Mean	Range	Mean	Range	Mean	Range
Basal leaf length	11	**13.3**	5.1-32.7	22.3	8.4-38.5	4.6	3.2-5.1
Basal leaf width	3	22.3	12.0-37.0	35.0	8.0-52.0	17.2	3.4-44.5
Basal leaf area	3	23.7	8.0-51.0	27.4	8.0-52.0	17.5	3.2-45.5
Green weight	3	32.3	12.0-60.0	27.2	20.0-38.0	4.4	3.6-5.8
Dry weight	3	30.7	15.0-60.0	26.4	13.3-42.8	4.1	3.1-5.6
Growth type	4	24.8	9.0-47.0	**13.3**	3.6-21.4	3.9	3.2-4.2
Leaf colour	5	38.5	11.0-57.0	**25.6**	**11.5-30.9**	**3.3**	3.1-3.5
Crown rust resistance	14	21.8	9.0-48.0	32.4	7.1-95.8	7.5	5.3-9.6
Height et ear emergence	3	**9.3**	4.0-18.0	19.3	17.1-21.0	3.5	3.3-3.7
Spike length	3	28.6	9.0-57.0	27.4	15.4-44.2	3.5	3.1-3.8
Spikelet length	3	16.6	12.0-21.2	**20.9**	**12.1-27.4**	**3.3**	3.3-3.4
Spikelet number	14	25.8	13.8-37.3	29.2	13.3-55.2	8.5	5.1-12.3
Floret number	6	40.5	29.0-70.0	26.1	20.6-37.1	4.3	3.3-5.5
Flag leaf length	3	26.3	22.0-31.1	43.3	23.5-75.1	3.7	3.4-4.2
Flag leaf width	16	25.2	2.4-51.0	25.1	7.5-47.0	12.3	9.4-15.4
Flag leaf area	14	**12.0**	9.1-15.7	29.5	6.6-58.0	5.7	4.3-8.8
Green weight of tillers	6	31.2	5.0-56.9	26.2	2.3-77.8	4.0	3.7-4.9
Dry weight of tillers	6	19.4	5.0-30.1	29.0	2.3-66.2	3.8	3.4-4.0
Winter survival	4	35.5	4.0-57.0	36.1	13.7-68.6	4.5	3.3-5.5
Days to ear emergence	12	27.7	12.8-46.5	30.2	15.9-51.0	14.0	8.2-15.8
Presence of awns	9	30.7	2.0-64.0	**9.7**	2.4-11.7	32.9	29.4-41.9
Total	**145**	**24.4**	**2.0-70.0**	**26.7**	**2.3-95.8**	**9.0**	**3.1-41.9**

Shadow boxes - maximum values at P=0.05 for LSD test, bold - minimum values at P=0.05 for LSD test

Explained variation and major QTLs

The magnitudes of the QTLs effect ranged from medium to big relative to the trait differences between *L. multiflorum* and *L. perenne*. The average QTL explained 25% of observed phenotypic variation however, a great range of QTL magnitudes was observed both for each character and individual QTLs (Table 7.3). Aside from winter survival, the biggest proportion of phenotypic variance explained by a single QTL was again found for traits not interesting to breeders. For example, the average QTL controlling the number of florets or leaf colour explained around 40% of variation observed in BR3 x NZ15 population whereas this value was of 10% lower for weight of vegetative parts. Traits for which the lowest proportion of variation was explained included height at ear emergence and flag leaf length.

The average QTL spanned over 27 cM on the genetic map however, the QTL as small as 2.4 cM was found for the presence of awns. The biggest QTL (95 cM) controlled crown rust resistance and it covered nearly half of the first linkage group suggesting a lot of underlying genes. The maximum LOD values were correlated in a certain degree with a QTL size; smaller QTLs had bigger effect as indicated by maximum LOD values. Presumably, this was attributable to QTLs responsible for the presence of awns that were among smallest but with the maximum LOD values up to tenfold bigger than for the other traits. Indeed, it was not surprising because this trait is not a typical quantitative character and it is frequently controlled by one or a few major genes. On the other hand, the presence or absence of awns was not always so obvious in the BR3 x NZ15 population, and many intermediate types were observed. This again suggested the more complex inheritance and several QTLs identified confirmed these predictions.

Expectedly, the number, size and magnitudes of QTLs for some traits were very alike suggesting a single QTL with pleiotropic effect. This was true for characters estimated by recalculation from the others, for example basal and flag leaf areas obtained by the multiplication of the leaf length by the leaf width. Note, that QTLs for areas corresponded to QTLs for width but not for length. Likewise, QTLs for green and dry weight were similar with respect to their effects and size.

For nearly all traits there were one or a few QTLs of large effect, so called major QTLs, and several others with smaller effects (minor QTLs). Major QTLs explained threefold more phenotypic variance than minor genes (Table 7.4). If a threshold of 25% was employed nearly all quantitative characters were controlled by at least one major QTL. The exceptions were height at ear emergence, spikelet length and flag leaf area. By contrary, floret number was controlled only by major QTLs, each with large effect ranged from 29% to 70% of explained phenotypic variance. Likewise, the most QTLs responsible for leaf colour and winter survival were major genes. Note, that the number of major QTLs controlling crown rust resistance in the *L. multiflorum* x *L. perenne* population correlated well with the number of qualitative classes recognised in the intraspecific populations. The size of major and minor QTLs was very alike. Similarly, the LOD value generally did not correspond to a QTL effect.

Table 7.4. Comparison of major and minor QTLs responsible for 21 quantitative traits in *L. multiflorum* and *L. perenne*

Trait	Major QTLs		Mean phenotypic variance explained [%]		Mean QTL size [cM]		Mean maximum LOD value	
	N	%	Major	Minor	Major	Minor	Major	Minor
Basal leaf length	1	9.1	32.7	12.4	14.5	23.1	5.1	4.5
Basal leaf width	1	33.3	37.0	15.0	52.0	26.4	3.4	24.1
Basal leaf area	1	33.3	51.0	10.0	52.0	15.1	3.9	24.3
Green weight	2	66.7	42.5	12.0	30.8	20.0	4.7	3.7
Dry weight	1	33.3	60.0	16.0	23.2	28.1	5.6	3.6
Growth type	2	50.0	38.5	11.0	14.1	12.5	4.1	3.7
Leaf colour	4	80.0	45.3	11.0	24.2	30.9	3.3	3.3
Crown rust resistance	4	28.6	33.5	17.6	33.0	32.2	8.4	7.2
Height et ear emergence	0	0.0	-	9.3	-	19.3	-	3.5
Spike length	1	33.3	57.0	14.4	44.2	19.1	3.7	3.4
Spikelet length	0	0.0	-	16.6	-	20.9	-	3.3
Spikelet number	5	35.7	33.5	21.6	33.9	26.6	10.2	7.6
Floret number	6	100.0	41.8	-	26.1	-	4.3	-
Flag leaf length	2	66.7	28.5	22.0	75.1	27.4	3.8	3.6
Flag leaf width	7	43.8	35.4	13.9	33.2	18.8	13.8	11.1
Flag leaf area	0	0.0	-	12.0	-	29.5	-	5.7
Green weight of tillers	3	50.0	44.2	15.7	31.9	20.5	3.9	4.1
Dry weight of tillers	2	33.3	28.1	15.0	17.2	34.9	3.5	3.9
Winter survival	3	75.0	46.0	4.0	40.8	22.2	5.0	3.3
Days to ear emergence	5	41.7	42.6	16.8	19.6	37.7	12.5	15.0
Presence of awns	3	33.3	62.7	16.3	18.6	5.2	28.7	35.0
Total	**53**	**36.6**	**40.9**	**14.9**	**28.7**	**25.4**	**8.5**	**9.4**

Shadow boxes – Significant differences between major and minor QTLs for QTL size and maximum LOD value at P=0.05 for LSD test, differences between mean phenotypic variance explained were significant for all traits but flag leaf length

Additive and dominance effects

The QTLs controlling all traits demonstrated both additive and dominance effects irrespective they were minor or major genes. The multiple QTLs responsible for the majority of traits had usually a mixture of positive and negative additive effects (Table 7.5). Different signs of QTLs controlling a particular trait indicated that each parent contained a mixture of genes, some acting to increase the value of a given trait while others act to decrease the value of that trait. On the other hand, when all QTLs responsible for a given trait had the same sign, it was considered to be strong evidence in favour of the accumulation of genes with positive effect in one of the parents. Unfortunately, such situation was observed for no trait if all QTLs were taken into account. Nevertheless, when major and minor QTLs were analysed separately the common pattern was either the accumulation of major QTLs with the same sign (positive or negative) in one parent and the dispersion of minor QTLs or the opposite situation. For example, dry weight of vegetative parts was controlled by a single major QTL increasing the value of this trait and originating from the *L. multiflorum* parent in addition to two minor QTLs. Minor QTLs had different signs, hence those increasing dry weight were dispersed between *L. multiflorum* and *L. perenne*. An opposite relationship was observed for green weight. In that case major QTLs with positive effects were dispersed between parents. For two traits, crown rust resistance and dry weight of tillers both minor and major QTLs with a positive influence on phenotypes were dispersed between parents. In contrast, all major

QTLs responsible for basal leaf width and area as well as growth type had negative additive effect and they were accumulated in one parent whereas minor QTLs had positive additive effects and they were accumulated in the other parent.

Table 7.5. Mean additive and dominance effects of QTLs controlling 21 traits in *L. multiflorum* and *L. perenne*

Trait		Major QTLs				Minor QTLs			
		Additive		Dominance		Additive		Dominance	
		P	N	P	N	P	N	P	N
Basal leaf length		4.35	-	1.45	-	3.40	1.55	2.10	5.82
Basal leaf width		-	0.04	-	0.03	0.17	-	0.07	-
Basal leaf area		-	1.48	-	1.41	3.94	-	0.06	0.95
Green weight		13.89	11.57	32.05	-	-	23.33	-	9.59
Dry weight		2.89	-	3.25	-	5.60	5.74	1.19	3.84
Growth type		-	0.21	0.46	-	0.15	-	0.13	0.39
Leaf colour		0.15	0.35	0.44	0.58	0.95	-	-	0.43
Crown rust resistance		0.04	0.83	1.16	0.25	0.61	1.73	1.04	0.57
Height et ear emergence		-	-	-	-	3.51	3.05	1.54	-
Spike length		1.17	-	-	1.73	-	1.28	1.86	-
Spikelet length		-	-	-	-	-	0.47	0.49	3.91
Spikelet number		0.42	2.53	7.22	3.23	0.12	3.03	5.8	3.13
Floret number		0.91	0.37	0.88	1.21	-	-	-	-
Flag leaf length		1.12	1.37	1.04	-	0.99	-	-	1.12
Flag leaf width		-	0.15	0.23	0.14	3.59	1.45	0.17	1.88
Flag leaf area		-	-	-	-	0.08	1.11	2.93	2.29
Green weight of tillers		36.28	57.77	16.32	23.17	22.50	-	35.21	35.03
Dry weight of tillers		8.94	4.02	35.57	4.57	8.65	5.98	10.36	9.10
Winter survival		9.18	1.71	11.62	21.27	-	4.56	1.83	-
Days to ear emergence		-	3.48	8.19	4.40	0.30	3.53	8.28	4.70
Presence of awns		0.03	-	0.75	-	0.37	0.04	0.76	0.38
Total N° of QTLs	**N°**	**22**	**31**	**28**	**25**	**36**	**56**	**45**	**47**
	%	**41.5**	**58.5**	**52.8**	**47.2**	**39.1**	**60.9**	**48.9**	**51.1**

N- negative effect, P – positive effect

The additive effects were unlikely to explain all of the variation observed. Particularly that the large dominance effects were demonstrated for the majority of QTLs. Similarly to additivity, if all QTLs were taken together, dominant genes with a positive influence on phenotypes were dispersed between parents as indicating by different signs of the QTLs controlling the same trait. In comparison with additive effects, dominant QTLs, both major and minor were more frequently dispersed between parents. In addition to crown rust resistance and dry weight of tillers, the other four characters including green weight of tillers, spikelet number, flag leaf width and days to ear emergence were controlled by such QTLs. For several traits only minor QTLs were dispersed. Examples involved minor QTLs with positive influence on phenotypes that originated either from *L. multiflorum* or *L. perenne* and controlled length of basal leaf, dry weight, growth type, and the presence of awns. The opposite relationship i.e., dispersion of dominant major QTLs and association of minor ones was rarely observed. However, a cautionary point to these considerations should be made. The fact that sum of estimated magnitudes for some QTLs exceeded 100% suggested that QTLs did not act independently of each other and epistatic interactions controlled at least some of analysed characters.

7.3.4. Genomic distribution of QTLs in *L. multiflorum* and *L. perenne*

QTLs were located among all linkage groups (Figure 7.6 A-G). Unsurprisingly, a little more QTLs were detected on longer linkage groups (Table 7.6) than on shorter. The similar tendency was observed for major QTLs. The mean phenotypic variance explained by all QTLs located on different linkage groups was very alike. Nevertheless, a QTL size depended on a linkage group and the biggest QTLs were located on the three longest groups i.e., LG1 (214.2 cM), LG2 (183.7 cM) and LG4 (164.1 cM). In general the mean LOD value was similar for the majority of linkage groups. The only exceptions were LG3 and LG6 for which mean LOD values were significantly higher. This result was attributable to QTLs responsible for the presence of awns and days from spring rush to ear emergence that had extremely high LOD values. In addition, the QTL for basal leaf length with LOD value equal to 45.5 was detected on LG3.

Table 7.6. Distribution of QTLs among linkage groups of *L. multiflorum* and *L. perenne*

Linkage group	Total QTLs		Major QTLs		Mean phenotypic variance explained [%]	Mean QTL size [cM]	Mean maximum LOD value
	N°	[%]	N°	[%]			
LG1	23	15.8	7	13.2	12.7	34.6^{a}	7.81^{b}
LG2	24	16.6	11	20.8	16.2	30.8^{a}	7.94^{b}
LG3	21	14.5	9	16.9	16.4	15.5^{c}	13.48^{a}
LG4	26	17.9	10	18.9	14.8	31.4^{a}	7.42^{b}
LG5	19	13.1	6	11.3	18.8	28.1^{ab}	8.03^{b}
LG6	21	14.5	8	15.1	16.0	21.0^{bc}	11.39^{ab}
LG7	11	7.6	2	3.4	11.9	19.6^{bc}	6.55^{b}

^{abc}different letters mean significant differences at P=0.05 for LSD test

Densities of QTLs calculated as a number of QTLs per 1 cM were somehow different on each linkage group and they were not correlated with LG length. Indeed, the highest density was exhibited by groups of modest length as LG6 and LG3. Visual inspection of QTLs' map positions demonstrated clearly some level of overlapping (Figure 7.6 A-G). A good measure of overlapping was obtained if the total length of all QTLs on a given linkage group was divided by the LG length (Figure 7.7). The obtained value that could be described as a coefficient of overlapping showed the highest number of QTLs occupying the similar position on LG4. As many as five QTLs shared the same position and this calculation was in agreement with the visual inspection. Notwithstanding the fact that the lowest overlapping was found on the shortest linkage group, LG7, no correlation was observed between the linkage group length and the level of overlapping. Somehow lower overlapping was found for major QTLs but it simply resulted from fewer major QTLs.

In some cases overlapping QTLs shared exactly the same position concerning flanking markers and markers linked with them at maximum LOD value. In these cases, it was reasonable to speculate that overlapping QTLs represented the same genes with pleiotropic effect. This must have been true for QTLs controlling green and dry weight of tillers on LG2 and LG4, green and dry weight of vegetative part on LG3 and basal leaf width and area on LG3. In other cases, despite QTLs spanned over the same distance, the linked markers with the highest LOD value were quite different. Although pleiotropy could not be excluded, the

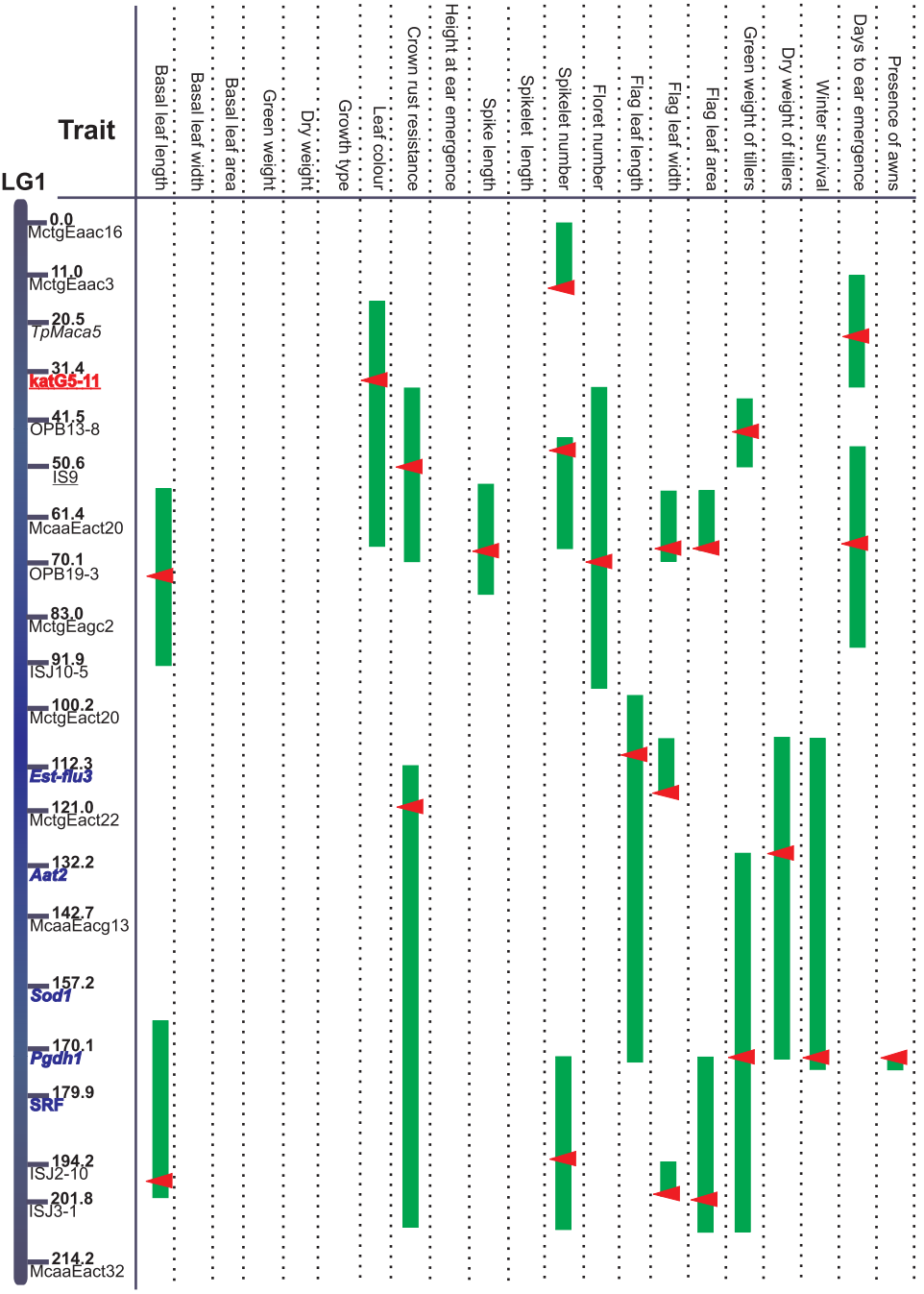


Figure 7.6A. The most likely position of the QTLs for 21 quantitative characters on LG1 of the genetic map of *L. multiflorum* and *L. perenne*
(Arrows within the confidence intervals indicate the position of the highest LOD score)

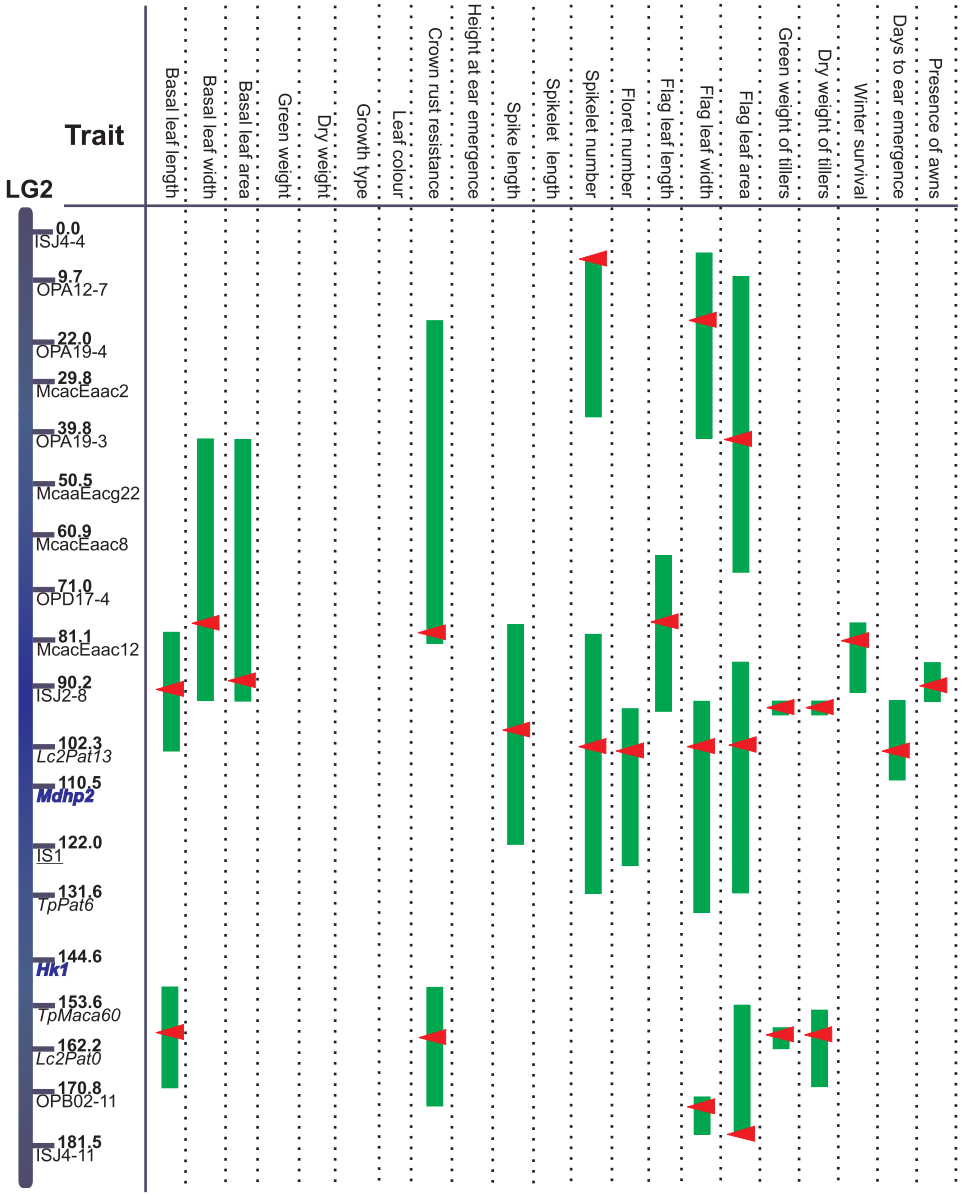


Figure 7.6B. The most likely position of the QTLs for 21 quantitative characters on LG2 of the genetic map of *L. multiflorum* and *L. perenne*
(Arrows within the confidence intervals indicate the position of the highest LOD score)

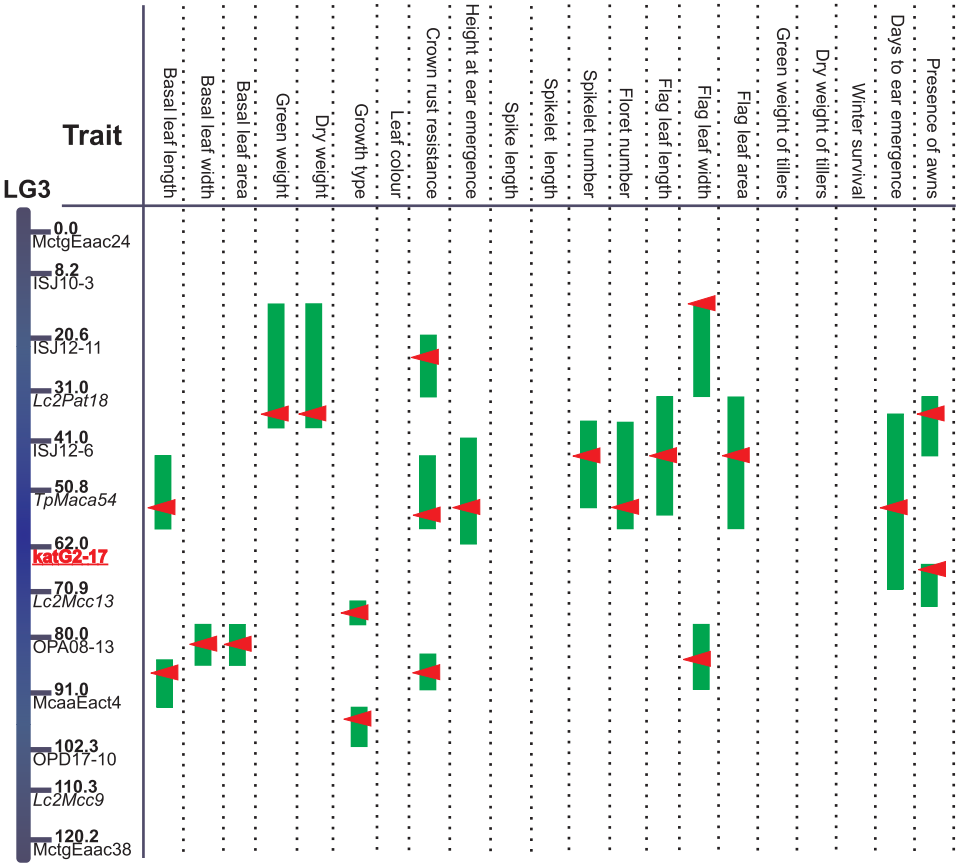


Figure 7.6C. The most likely position of the QTLs for 21 quantitative characters on LG3 of the genetic map of *L. multiflorum* and *L. perenne*
(Arrows within the confidence intervals indicate the position of the highest LOD score)

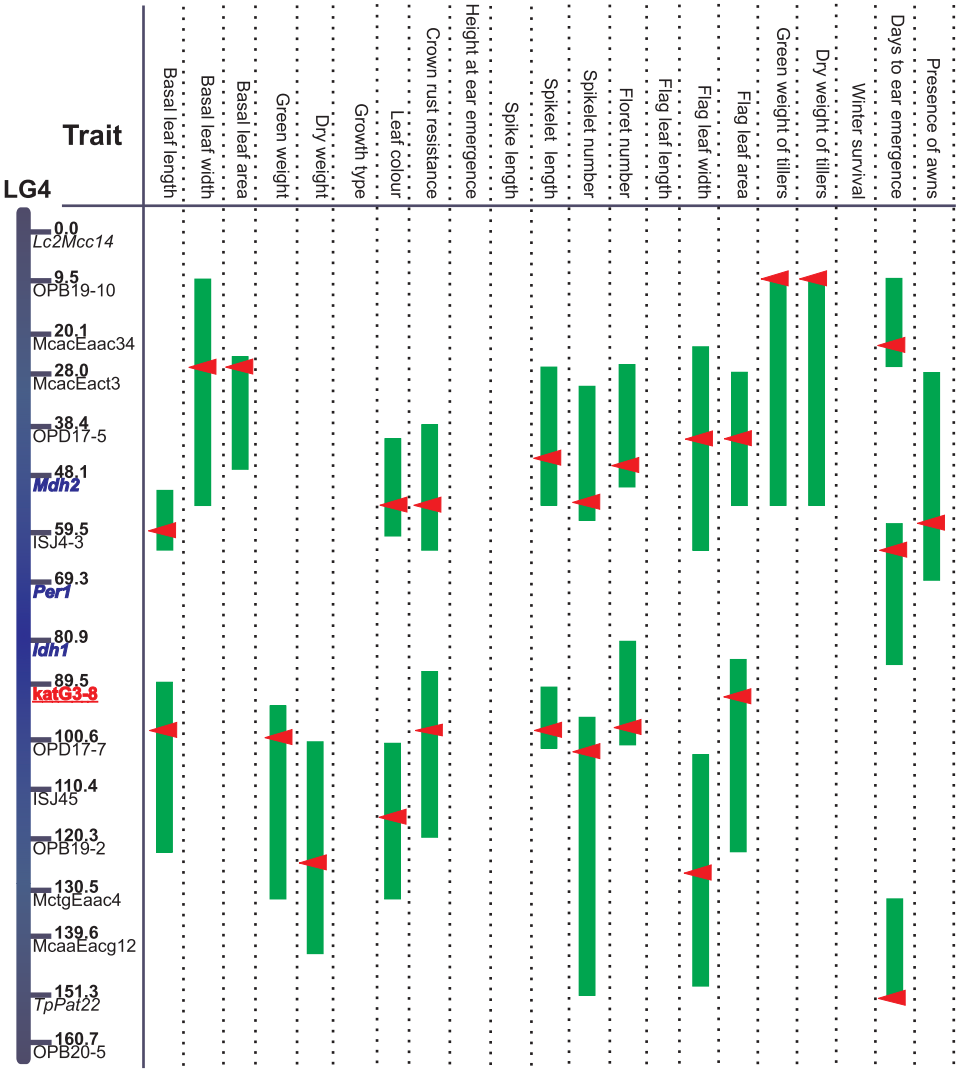


Figure 7.6D. The most likely position of the QTLs for 21 quantitative characters on LG4 of the genetic map of *L. multiflorum* and *L. perenne*
(Arrows within the confidence intervals indicate the position of the highest LOD score)

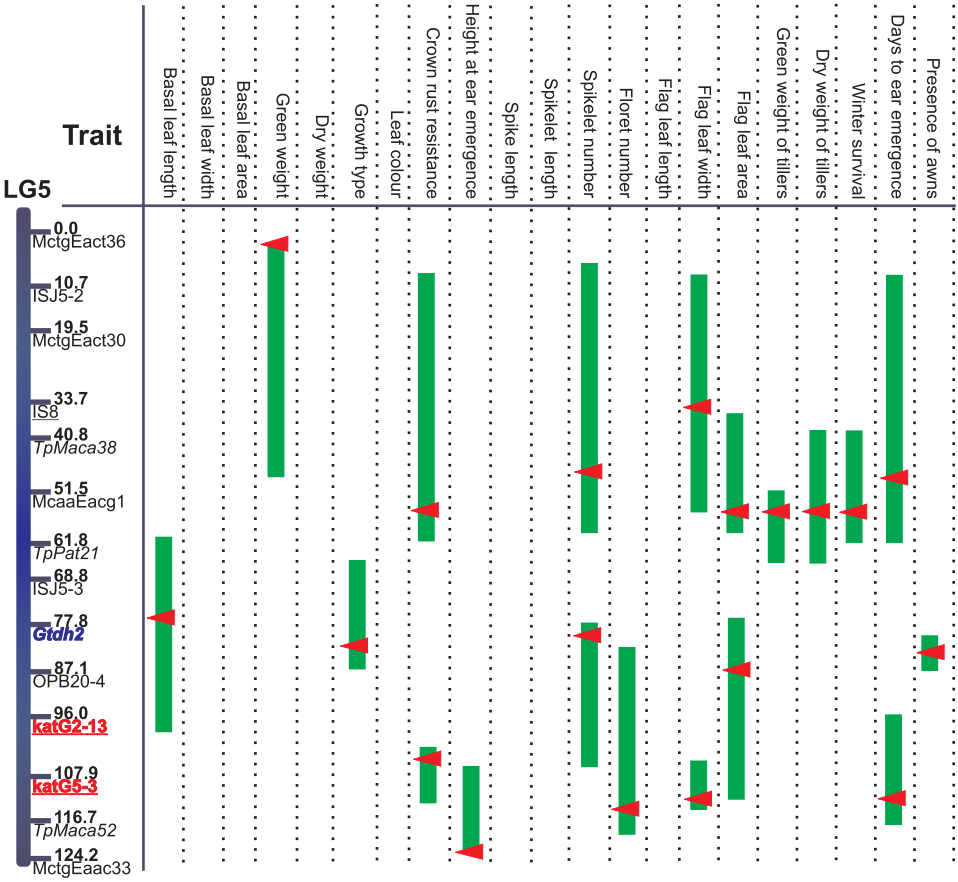


Figure 7.6E. The most likely position of the QTLs for 21 quantitative characters on LG5 of the genetic map of *L. multiflorum* and *L. perenne*
(Arrows within the confidence intervals indicate the position of the highest LOD score)

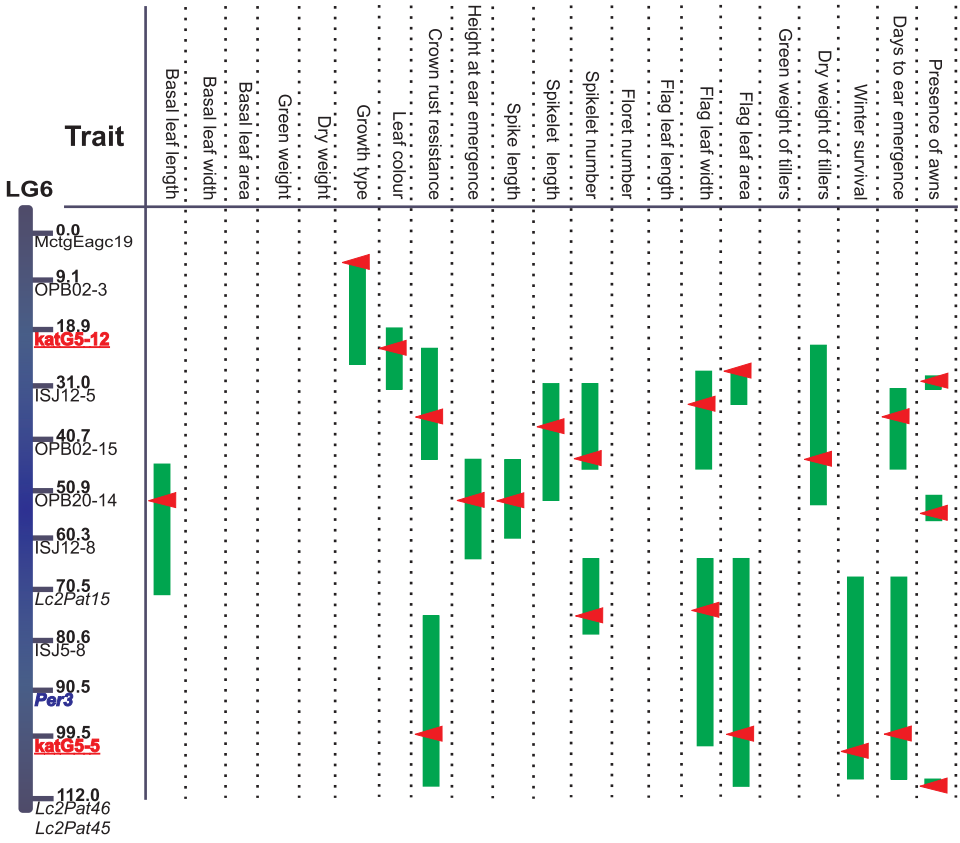


Figure 7.6F. The most likely position of the QTLs for 21 quantitative characters on LG6 of the genetic map of *L. multiflorum* and *L. perenne*
(Arrows within the confidence intervals indicate the position of the highest LOD score)

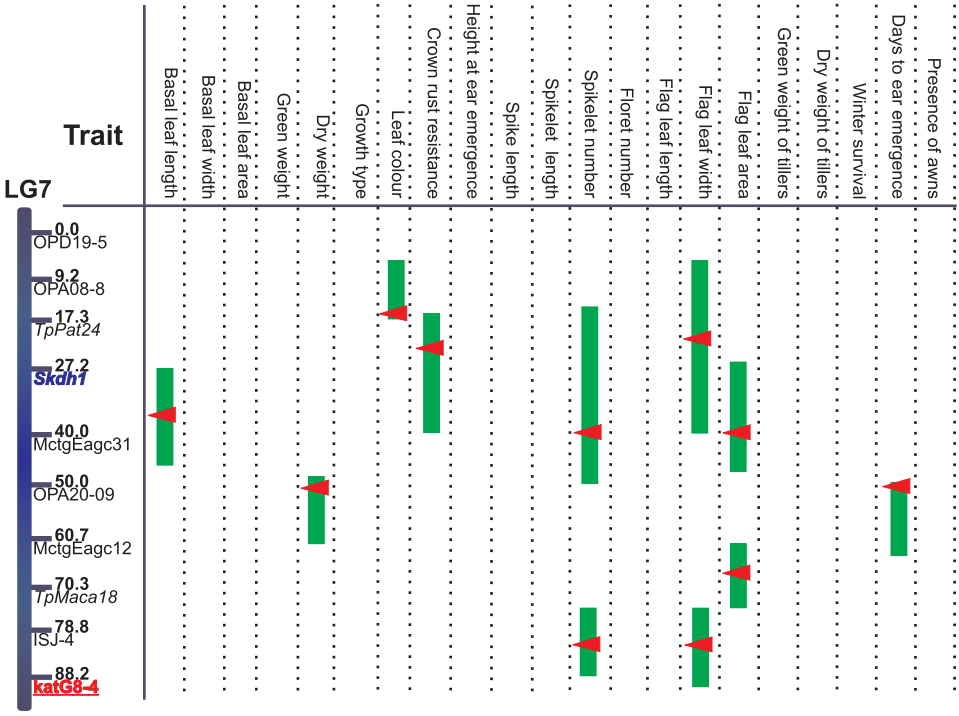


Figure 7.6G. The most likely position of the QTLs for 21 quantitative characters on LG7 of the genetic map of *L. multiflorum* and *L. perenne*
(Arrows within the confidence intervals indicate the position of the highest LOD score)

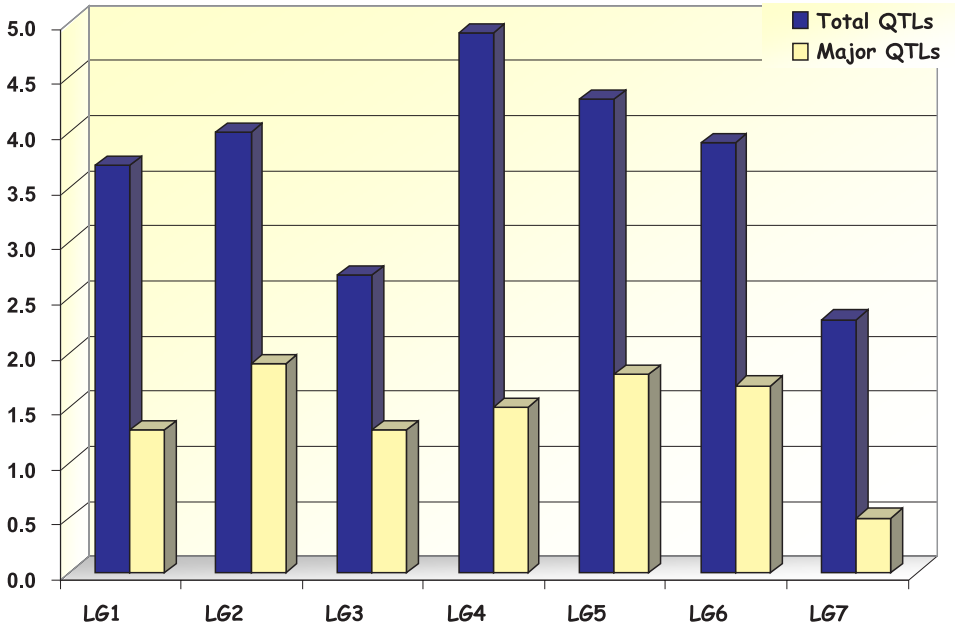


Figure 7.7. Coefficient of QTL overlapping in the F_2 population of *L. multiflorum* and *L. perenne*, BR3 x NZ15
(Coefficient of overlapping was calculated as the ratio of total length of QTLs on a LG in cM to the total length of a LG)

hypothesis about many linked genes seemed more plausible all the more these QTLs often controlled different traits. Examples involved QTLs responsible for crown rust resistance and leaf colour that shared the similar position on LG1, spikelet number and floret number on LG2, LG3, LG4, dry weight of tillers and winter survival on LG5 and several others.

The QTLs for majority of characters were distributed among all linkage groups and no significant clustering was observed. Even though two QTLs controlling the same trait were found on a single linkage group this phenomena was related only with the most abundant QTLs that were present in multiple copies on nearly all linkage groups. If vegetative characters were considered together (basal leaf, weight of vegetative part), they were predominantly observed on LG2, LG3 and LG4. QTLs controlling green and dry weight of tillers usually went together with the exception of one QTL for each character that was found on LG1 for green weight and on LG6 for dry weight. Additionally, they were associated with winter survival and days to ear emergence on LG2 and LG5. Similarly QTLs for leaf area both basal and flag leaf were connected with QTLs for leaf width but not necessarily with these for leaf length. It was surprising because leaf area was obtained as the multiplication of leaf length by leaf width. Another unexpected result was the co-location of two quite different traits such as the number of spikelets per a spike and the number of florets per a spikelet. These two traits went together on four linkage groups. Sometimes they were also associated with the spike and flag leaf length. It is worthy to note that the QTLs for the presence of awns were hardly correlated with the QTLs corresponding to the other spike characters.

When all linkage groups were analysed together with respect to all characters, some patterns could be found. For example, it was possible to find QTLs responsible for nearly all analysed characters on LG4. Conversely, almost no QTL controlling vegetative growth was found on LG1 and LG6. The LG7 was rather poor in QTLs. Such unequal distribution suggested that some chromosome regions were particularly related with differences between *L. multiflorum* and *L. perenne* and they might play a significant role in their further diversification.

The distribution of QTLs within each linkage group varied from one to another. On some groups QTLs were detected rather closer to ends (LG1, LG4, and LG5) while on the others near the centre (LG2, LG3). If we turn instead to linkage between QTLs and markers, there was a clear tendency to more frequent associations with some groups of markers than the others (Figure 7.8). If markers flanking or linked with QTLs (those with maximum LOD value) would be simply a random sample then the proportion of a given marker type among all QTL flanking or linked markers should be equal to the proportion of that marker type among all mapped markers. For example, if RAPD markers constituted 26% of all mapped markers, they should also make circa 26% of markers flanking and linked with QTLs. Surprisingly, the above rule was not true and markers flanking or linked with QTLs were not a random sample. It is somehow strange that the representation of ISJ markers was lower than expected from random distribution. These markers are complementary to exon-intron sequences and one could imagine that they will mark coding sequences including QTLs. The lower number of enzymatic loci tightly linked with QTLs seemed logic. In fact, their function is known; they encode enzymes that are not necessarily associated with quantitative characters. The same explanation could be true for katG markers that were shown to be correlated with enzymatic loci (Chapter 6). However, both enzymatic loci and katG markers more often flanked QTLs than expected from random distribution. Likely, it was a manifestation of the predominant distribution of both enzymes and QTLs in gene-rich regions. The most dramatic findings came from AFLP, RAPD and transposons demonstrating that AFLP markers were underrepresented among these correlated with QTLs while RAPD and both types of transposons, *Tpo1* and *Lolcopia2* were favoured. Overrepresentation of SSR markers, that could also be transposon sequences as discussed in Chapter 6, made plausible that repetitive and transposable sequences were correlated with QTLs.

7.4. DISCUSSION

Knowledge about the underlying genetics is critical to understanding how quantitative characters evolve over time. Differences in quantitative characters if there are no reproductive barriers between two populations or taxa as in a case of *L. multiflorum* and *L. perenne* do not entitle to rank them as biological species. Nevertheless, they provide foundation for potential diversification. Generally, quantitative variation is non-random and correlated with adaptation to environment. It can be speculated, considering results from previous chapters that perenniality of *L. perenne* has evolved as an adaptation to harsh environment during Tertiary and Quaternary glaciations. Floral morphology in two *Mimulus* species that is controlled by several QTLs is strongly correlated with adaptation to two different pollinators,

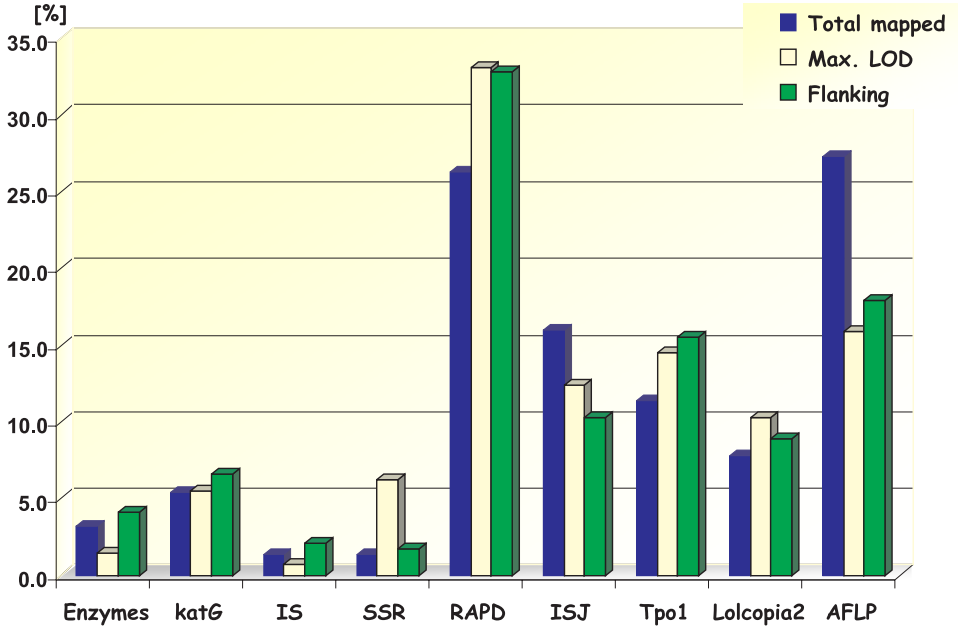


Figure 7.8. Markers with maximum LOD values (linked) and markers flanking QTLs in relation to total number of markers associated with QTLs

bees and hummingbirds (Schemske and Bradshaw 1999). Likewise, adaptation to different pollinators may be responsible for differences in floral morphology between two subspecies of *Solidago virga-aurea* (R. Zielinski, personal communication). The first subspecies, *S. virga-aurea* ssp. *virga-aurea*, typical of plains and lower parts of mountains is about 70 cm high and has small capitulum of 0.7 cm in diameter. In higher mountainous parts, *S. virga-aurea* ssp. *alpestris* is more common. It is shorter (30 cm) and has twofold bigger capitulum. Nevertheless, in between they are crossing giving rise to the wide spectrum of hybrids. Another nice example includes *Polygonatum verticillatum* that is short in mountain areas and tall in lowlands and this morphology does not change in experimental conditions (M. Szczecinska, personal communication). Numerous examples of dramatic changes in morphology during domestication processes are another manifestation of the same phenomena. Although all mentioned examples deal with fully interfertile populations or subspecies, they illustrate very well differences in quantitative characters that over long-term periods can contribute to speciation. Considering the role of quantitative characters in evolution, the most important is to understand that all these changes usually predate the acquisition of intrinsic reproductive barriers although they can be accompanied by prezygotic barriers (geographic, behavioural). On the other hand, the polymorphism in quantitative characters can be maintained in populations by abiotic and biotic stresses without producing new species. Thereby, any differences in quantitative characters should be understood only as potential possibility of speciation without any assurance that it will proceed. This conclusion should be kept in mind when considering the evolutionary processes in *L. multiflorum* and *L. perenne*.

7.4.1. Genetic basis of heterosis in *L. multiflorum* and *L. perenne*

Heterosis or hybrid vigour belongs to one of the most important phenomena related with differences in genes responsible for quantitative characters at a species level. It is defined as increase in size, yield and disease resistance in first generation hybrids in comparison with homozygous parents (Shull 1952). Unfortunately, owing to complicated genetic basis much controversy has dominated the debate of its role in evolution. One often expressed view, supported by many examples from geographically distant races, is that the more diverse are parents the greater heterosis is observed. Such association is clearly visible when performances of both interspecific hybrids from crosses between *L. multiflorum* and *L. perenne* are compared. Heterosis is expressed for about 33% of traits if genetic similarity between parents is 0.588 (BR3 x NZ15) but it is not observed at all if genetic similarity increases to 0.754 (HU5 x BO2). However, some authors go further and correlate heterosis predominantly with closely related species (Rieseberg et al. 2000). They regard heterosis as the major contributor to the evolutionary success of allopolyploid species, clonal hybrid lineages and hybrid species. Imagine, under the above hypothesis parents of both intraspecific crosses analysed in the current studies should be classified as closely related, yet distinct species owing to the high level of heterosis. The results from crosses between *L. multiflorum* and *L. perenne* would be even more astonished. Parents from one cross (BR3 x NZ15) would be classified as closely related taxa whereas from the other (HU5 x BO2) would represent one species. This sobering example demonstrates how much incomprehension and little knowledge are about early stages of evolutionary divergence.

An emerging consensus seems to be that the correlation between hybrid performance and genetic distance of parents is limited and when too many differences are accumulated, the reduction of fertility or viability is observed. Such situation is typical if two species are crossed. Even they are closely related and reproductive barriers are incomplete (Polok 1996). Cultivated rice is one of the most diverse crops with morphologically distinct japonica and indica forms. Despite significant genetic diversification, hybrids between them are fertile and exhibit strong heterosis effect supporting their status as a single species *Oryza sativa*. Ultimately, they are classified as subspecies, *O. sativa* ssp. *japonica* and *O. sativa* ssp. *indica* (Zhao et al. 1999). From the above perspective it is clear that heterosis demonstrated by the F_1 hybrid derived from the cross between *L. multiflorum* x *L. perenne* (BR3 x NZ15) is a strong argument for lowering the species status of both taxa.

Despite many examples supporting the correlation between genetic distance of parents and the level of heterosis (also from the current studies) there are at least as many contradictions. Molecular marker-based genetic distances are not suitable for predicting hybrid performance in maize (Benchimol 2000), rice (Zhao et al. 1999), wheat (Liu et al. 1999) as well as in *L. multiflorum* and *L. perenne*. Heterosis has been observed for several traits in both intraspecific crosses in spite of high genetic similarities (0.842-0.870) in comparison with interspecific ones (0.588-0.784). Hybrids from intraspecific crosses have been apparently superior with respect to the level of heterosis for dry weight of tillers (*L. perenne*) and spike length (*L. multiflorum*). The discrepancies obtained for inter- and intraspecific crosses, suggesting from one side the correlation between genetic distance and heterosis (interspecific crosses), and from the other, the lack of it (intraspecific crosses)

make plausible that heterosis in *L. multiflorum* and *L. perenne* resulted rather from interactions between specific genes than from the overall heterozygosity.

The view emerging from the analysis of QTLs in the *L. multiflorum* x *L. perenne* population, BR3 x NZ15 is that QTLs associated with heterotic traits and with positive effects on phenotypes are dispersed between parents. Similar conclusions can be drawn by inspecting parental phenotypes in the other populations. Floret number per a spikelet is just an example of a trait for which the hybrid exceeds the better parent of 35%, but the parental values are very alike and rather low in comparison with the other crosses. This character is an important separator of *L. multiflorum* and *L. perenne* in many dichotomous keys. The observed heterosis resulting in values similar to those in *L. multiflorum* seriously questions the utility of the number of florets for taxonomic purposes. Notably, six major QTLs are responsible for this trait in the *L. multiflorum* x *L. perenne* F_2 population. The *L. perenne* hybrid performance makes plausible that all of them also segregate in this species and presumably, QTLs increasing the number of florets are dispersed between parents. According to the dominance theory the dispersion of alleles with positive influence on a phenotype is sine qua non of heterosis (Polok 1996).

Referring to the possible genetic basis of heterosis it is likely that dominance with additive and non-additive interactions between loci are the most important in *L. multiflorum* and *L. perenne* hybrids. The dominance effects for the majority of QTLs associated with heterotic traits in the *L. multiflorum* x *L. perenne* population (BR3 x NZ15) in addition to asymmetric distribution of F_2 individuals support the above opinion. This hypothesis has strong roots in earlier wide explorations of mechanisms responsible for heterosis in crosses between barley mutants. The homozygous lines derived from heterotic crosses, performing as good as heterotic hybrids and significant estimations of parameters informing about additive gene actions and interactions between homo- and heterozygous loci prove that the interaction between dominant loci is the most important mechanism underlying heterosis. In the most striking examples it is possible to connect heterosis with recessive epistasis. Another important outcome from barley studies is that even a few genes differentiating mutants from their parental cultivars are suitable to express heterosis in hybrids provided the effects of all dominant genes and interactions have positive influence on phenotypes. To exemplify, most heterotic traits involve from two to six genes or groups of linked genes, however for some traits ten genes can be found (Polok 1996, Polok et. al. 1997). The present data for *L. multiflorum* and *L. perenne* are in good agreement with those cited above. Heterotic traits can be controlled by either a few QTLs or quite a lot. For instance, spike length, flag leaf length in the *L. multiflorum* x *L. perenne* hybrid, BR3 x NZ15 are associated with only three QTLs, the number of QTLs responsible for green and dry weight of tillers is twofold bigger (six), whereas spikelet number and flag leaf width are controlled by as many as 14-16 QTLs both with major and minor effects.

In summary, there are no differences with respect to the occurrence, magnitude and mechanisms underlying heterosis in inter- and intraspecific *Lolium* crosses. It demonstrates clearly that *L. multiflorum* and *L. perenne* perhaps represent two extreme phenotypes but still constituting a single biological species. On the other hand, the heterotic hybrids can have higher adaptive values that under certain circumstances might lead to rapid adaptation to new environments and result in future diversification.

7.4.2. QTLs involved in the evolution of *L. multiflorum* and *L. perenne*

A glance at teosinte, a grass that grows robustly in uncultivated areas of Mexico has to evoke astonishment. Although maize is a descendant of teosinte, the seeds and plants look much different. The teosinte plant produces several tillers, whereas maize has one main stem. Teosinte stalks each bear several ears, but they are much smaller than those of maize. Nevertheless, thousands of years of mutations and human selection catalyzed the evolution of teosinte from a wild grass into modern corn. Despite of these differences teosinte and maize are a single species. The differences between maize and teosinte are governed by a few major genes, a few blocs of multiple linked genes plus a larger number of small effect loci. They are scattered throughout the genome. For example, a QTL on the first chromosome causes the ears of corn to be big and to grow on a few short branches. A gene on the second chromosome causes more rows of kernels to grow, yielding more food per corn plant (Doebley 2004). The striking example of maize demonstrates how QTL mapping can open "a door" into searches for the "footprints" of domestication and evolution.

A glance at *L. multiflorum* and *L. perenne*, grasses that grow predominantly in Europe in cultivated and semi-natural areas has to evoke astonishment as well. Although they are classified by botanists as distinct taxa, they are nearly unrecognizable, they possess the same gene pool, similar genomes (Chapter 3-6), karyotypes (Naylor 1960) and they are fully interfertile. The broad genetic analyses presented in the proceding chapters have demonstrated that the paradox of *L. multiflorum* and *L. perenne* could best be explained by regarding them, similarly to teosinte and maize as a single species. This raises next questions. Can one demonstrate that discrete shifts in morphology results from intraspecific variation? How many genes are involved in minor differences between them? What are these genes? What is the influence of human activity (breeding) on their diversification? The current pioneering studies on QTLs potentially involved in morphological differences between *L. multiflorum* and *L. perenne* confirm little genomic diversification of these species but also seem to demonstrate that they begin to evolve and it can be expected that they will follow in teosinte and maize or many other domesticated species footsteps. All the more there are old conceptions that perennial forage grasses are not domesticated with the single exception...*L. multiflorum*. It was developed by unconscious selection prior to the twelfth century in the Lombardy and Piedmont plains of Italy (Beddows 1953). The domestication of Italian ryegrass likely was associated with the domestication of livestock for agriculture. Ryegrass used to exist in "hybrid swarms" with perennial and Italian phenotypes as opposite extremes of a continuum. Hay harvesting, and reseeding with shattered seeds, resulted in the selection of tall, sparse-tillered, semi-annual phenotype that has since been elevated to species status (Casler and Duncan 2002). This theory is in perfect agreement with the results from the previous chapters suggesting that *L. multiflorum* is younger than *L. perenne* and stems from the "complex species". This means that in a case of *L. multiflorum* and *L. perenne* we are witnessing the same sort of processes which have entailed the diversification of teosinte and maize for a few thousand years. At this point one can ask: Why are they not so different? Since they have only hundreds of years to evolve whereas maize and teosinte - thousands. Thereby, the previous questions should be transformed. What are the genes and how many of them are involved in the domestication of *L. multiflorum*? And the answer is - a lot of genes.

A huge number of 145 QTLs are responsible for differences in 21 quantitative traits between *L. multiflorum* and *L. perenne*. A comparable huge number of QTLs are related with the domestication of *T. dicoccoides* (Peng et al. 2003). More QTLs are predominantly associated with traits that likely have not been under strong selection pressure generated by human activities. The examples involve basal leaf length (11 QTLs), flag leaf width and area (16 and 14, respectively) and spikelet number (14). In contrast, the characters that might have been selected during the domestication of *L. multiflorum* are governed by just a few QTLs (3-6). These QTLs are both minor and major in respect to explained phenotypic variances. Similarly, in *Q. robur* and *Q. petraea* more QTLs are related with traits of low economic value (Saintagne et al. 2004).

Most traits appear to be controlled in part by at least one major QTL explaining more than 25% of the total phenotypic variance. Some of them explain even more than half of the phenotypic variance. Green and dry weight of vegetative parts, floret number, and winter survival are just a few examples. This implies that either single genes of individually large effects or clusters of linked genes with a large summary effect can play a role in diversification of *L. multiflorum* and *L. perenne*. In addition only a modest number of mutations in QTLs may be required for their diversification. The pattern demonstrating that many morphological characters in plants are controlled by genes of large effects is common both in domesticated crops and wild species. The most famous major QTL is *teosinte branched1 (tb1)* separating teosinte from maize (Hubbard et al. 2002). However, many other examples are known such as a cluster of eight major QTLs associated with the domestication syndrome factor in *T. dicoccoides* (Peng et al. 2003) or several major QTLs controlled panicle and spikelet structure in rice (Paterson 2002). Among wild plants, each trait responsible for differences in flower morphology between *M. lewissii* and *M. cardinalis* is associated with at least one major QTL (Bradshaw et al. 1998). The most plausible hypothesis explaining the prevalence of major QTLs in domesticated plants is strong artificial selection followed by fixation of QTLs with large effects.

All QTLs involved in *L. multiflorum* and *L. perenne* morphological evolution are scattered throughout the genome and they have no strong tendency to clustering in comparison with such crops as maize, rice and wild emmer wheat. The initial events of *Lolium* domestication were quite recently and there has not been enough time to fix all QTLs. Moreover, the selection pressure has surely not been so strong like in cereals. However, some regions most strongly associated with differences between *L. multiflorum* and *L. perenne* can be observed. In total nine regions predominantly related with domestication syndromes can be distinguished on six linkage groups. Two groups are identified on LG1, LG4 and LG6, and one group on LG2, LG3, and LG5. None domestication syndrome region is present on LG7. Each of two regions on LG4 is connected with different characters; the first lying on the more distal part is responsible for differences in vegetative traits, whereas the second located in the proximal part controls generative characters including spike morphology and weight of tillers. It is likely, that each group is connected with different arms of a chromosome. The other linkage groups carrying two domestication syndrome regions, LG1 and LG6 are associated with generative traits and winter survival. The regions controlling mostly generative characters are also located on LG2 and LG5. By contrary, the LG3 region governs the diversification of both morphological and vegetative traits. It can be assumed that these syndromes are related with

gene-rich regions. In the progenitor of wheat, *T. dicoccoides* approximately 70 domestication QTLs are nonrandomly distributed among and along chromosomes. They are clustered into seven domestication syndrome factors, each affecting 5-11 quantitative characters and showing remarkable association with gene-rich regions (Peng et al. 2003).

A tremendous number of QTLs responsible for differences between *L. multiflorum* and *L. perenne* show the exceptional possibilities for further their evolution. Surely, not all QTLs are involved in the process of their domestication. However, the connection of some of them seems to be apparent. For instance, height at ear emergence, probably selected as the adaptation to hay harvest, is controlled by three minor QTLs, of which two are located within domestication syndrome regions together with generative characters (LG3, LG6) and the third is a solo QTL on LG5. The lack of major QTLs may indicate the narrow genetic background for this trait in modern cultivars and ecotypes. Similarly, spike and spikelet length that could be unconsciously selected towards bigger values are under control of three, mostly minor QTLs located within domestication syndrome regions. Nevertheless, each trait evolves rather independently for the reason that responsible QTLs are rarely co-located.

Six QTLs are responsible for the green and dry weight of tillers, while only three are associated with green and dry weight of vegetative parts. The QTLs related with weight of tillers and vegetative parts are not co-located that means two types of selection should have taken place during the evolution of *L. multiflorum* and *L. perenne*. First, it has been the selection towards higher yield of hay during the domestication of *L. multiflorum* and this has apparently been associated with changes in QTLs controlling weight of tillers. Half of all QTLs responsible for this trait are major QTLs. They are distributed among six linkage groups with prevalence on LG1 and LG2 within domestication syndrome regions. Second, the selection towards the resistance to grazing in *L. perenne* may have been, in a certain degree, the selection towards higher green weight. Grasses under continuous grazing should have the ability to grow rapidly to stay alive and if they are allowed to grow continuously without grazing for a longer period, like in the experimental conditions, they produce higher weight of vegetative parts. The major QTLs governing the evolution of green and dry weight of vegetative characters are mapped within the domestication syndrome regions on L3 and another two are clustered with the others QTLs responsible for vegetative characters on LG4.

Winter survival is another trait connected with domestication. The shift from perennial to annual forms is of a great importance for the evolution of many crops. It is usually connected with the lost of rhizomes that permit to survive in harsh environment. This character in *Lolium* is mostly controlled by major QTLs (three major and 1 minor QTL) with strong additive and dominance effect, all mapped within domesticated syndrome regions (LG1, LG2, LG5, LG6). At least some of these QTLs may be connected with two key genes responsible for rhizomatousness that diverged from a common ancestor of Poaceae about 50 MYA. In *Oryza longistaminata* two dominant genes *Rhz*2 and *Rhz*3 are responsible for many rhizome traits. They also contribute to regrowth and persistence of perennial grasses. The dominant nature of both genes causes that a single mutation resulting in loss of function shuts off rhizome expression. The close correspondence between rhizome genes/QTLs in distantly related rice and sorghum suggests that the convergent evolution of independent mutations at corresponding loci may be responsible for the domestication of many grasses (Hu et al. 2003).

The floret number per a spikelet is another important trait in the evolution of *L. multiflorum* and *L. perenne* that is controlled by six major QTLs. They are mostly located within regions associated with the domestication (LG1, LG2, LG3, and LG4) and they are very often co-located with QTLs associated with the spikelet number. Such location champions the selection of this trait as the selection toward higher yield. Indeed more florets mean more seeds. It can be also speculated that additional selection pressure has been related with the elevation of *L. multiflorum* to a species rank followed by the separation of *L. multiflorum* from *L. perenne* based on the floret number. Then it would be an example of a trait which selection has been promoted by taxonomists.

One final point about evolution of *L. multiflorum* and *L. perenne* is an intriguing discovery of obvious association between QTLs and transposon-based markers confirming the possible role of mobile genetic elements in morphological evolution of *Lolium*. QTLs are more frequently flanked or linked both with the DNA transposon, *Tpo1* and the retrotransposon, *Lolcopia2*. Although this association can be coincidental it is in agreement with the line of evidences from the other plants. Eight from ten yield enhancing QTLs from *O. rufipogon* and *O. sativa* contain transposons from CACTA family and both classes of retrotransposons, *Ty1-copia* and *Ty1-gypsy* (Reddy et al. 2006). A nonautonomous *Mutator*-like transposon is also present in the FLC allele responsible for flowering time in *A. thaliana* (Gazzani et al. 2003). The data again demonstrate that transposons make genomes a dynamic structure responding to various evolutionary forces. Although we are far to understand the role of transposons, the domestication of *L. multiflorum*, that is in action, can be a good experimental field to study it. And in the end, the current studies including all presented within Chapters 2-7, have provided the strongest support that *L. multiflorum* is a domesticated form of *L. perenne*. Therefore, implementing the same philosophy like for maize and teosinte, or indica and japonica forms in rice, it can be proposed to classify them, in agreement with the Integrated Taxonomic System of the USA (2007), as subspecies i.e., *L. perenne* ssp. *perenne* for a more primitive form and *L. perenne* ssp. *multiflorum* for a domesticated form.

7.5. CONCLUSIONS

1. The lack of differences with respect to the occurrence, magnitude and mechanisms underlying heterosis in intra- and interspecific *Lolium* crosses, confirms the status of *L. multiflorum* and *L. perenne* as a single biological species.
2. Heterosis in intra- and interspecific crosses results from dominance and gene interactions and it is not associated with genetic distance between parents. The number of QTLs responsible for heterotic traits depends on a trait and ranges between three QTLs up to 16.
3. *L. multiflorum* is a domesticated form of *L. perenne* that was selected in the Middle-Ages, and after that elevated to species status. The domestication process is controlled by many major QTLs in addition to several minor QTLs that are located within nine domestication syndrome regions on six linkage groups.

4. In total 145 QTLs govern the *L. multiflorum* and *L. perenne* evolution however, the most important traits related to domestication are governed by 51 QTLs. These traits involve height at ear emergence, spike and spikelet length, spikelet number, floret number, green and dry weight of tillers, green and dry weight of vegetative parts and winter survival.
5. Domestication of *L. multiflorum* and diversification from *L. perenne* is at least partially connected with transposons. This finding confirms the role of mobile genetic elements in the early evolution of *L. multiflorum* and *L. perenne*.
6. Implementing the same philosophy as it is done for maize and teosinte, or indica and japonica forms in rice, it is proposed to classify *L. multiflorum* and *L. perenne* with a rank of subspecies i.e., *L. perenne* ssp. *perenne* for a wild form and *L. perenne* ssp. *multiflorum* for a domesticated form.

8. MOLECULAR PHYLOGENY OF THE GENUS *LOLIUM*

8.1. INTRODUCTION

Once evolutionary relationships between *L. multiflorum* and *L. perenne* are resolved the phylogenesis of the whole genus *Lolium* can be reconstructed. First, it should pointed out that all data presented within chapters 2-7 demonstrate clearly that taxonomic rank of *L. multiflorum* and *L. perenne* should be lowered to the subspecies level. Thereby both taxa should be reclassified following the Integrated Taxonomic System of the USA (2007), in which they have names *L. perenne* ssp. *multiflorum* and *L. perenne* ssp. *perenne*. Consequently, this classification is used in all subsequent parts. Nevertheless, to make the text more readable and to avoid repeating the long subspecies names over and over again the abbreviations are used, *multiflorum* for *L. perenne* ssp. *multiflorum* and *perenne* for *L. perenne* ssp. *perenne.*

Phylogenesis is the sequence of events involved in the evolutionary development of a taxonomic group. Methods of reconstruction of phylogenetic trees were developed independently in numerical taxonomy (phenetic approach) for morphological traits and in population genetics for allele frequency data. Until then years of studies had been necessary to accumulate experience and classify species into a hierarchical system. The principles guiding phenetic approach are classifications based on overall similarity (or distance) between taxa measured by strictly defined rules and using as many characters as possible. Evolutionary relationships are presented graphically as phylogenetic trees or networks. External nodes are extant units; and internal nodes are deduced ancestral units. Branch lengths reflect the number of evolutionary changes along each ancestral-descendant pathway. The major advantage of numerical taxonomy is that a potential danger of authoritative selections of characters is limited. An alternative philosophy has been proposed by the cladistic school, according to which phylogeny should be appraised not by overall similarity, but rather by a set of similarities attributable to some shared-derived traits (Nei and Kumar 2000; Avise 2004). The basic difference between phylogenetic and cladistic approach is that the former tends to focus on branch lengths whereas the latter on the branching component. Indeed, there is a little justification for the differentiation of both approaches, first, because cladistic methods come close to phenetic ones especially as applied to large datasets. The second, more serious difficulty in all phylogenetic methods, either phenetic or cladistic is the assumption that the characters analysed are “independent”. This assumption is critical for most mathematical algorithms but unfortunately, it is probably not valid for the majority of morphological characters. Thus, although phenograms and cladograms based on morphological traits have

been generated for many taxonomic groups, they have not produced a clear-cut picture and reconstructed phylogenetic trees have often been controversial.

The first classification based on molecular data was published by "Angiosperm Phylogeny Group" in 1998 with aim to compile published phylogenetic trees into a hierarchical system at and above the level of family (Savolainen and Chase 2003). Ironically, notwithstanding this fact, molecular analyses are often treated as additional data, with lower "taxonomic" value in comparison with morphology, especially at the species level. This philosophical pillar still holds in the majority of taxonomic systems of plants, in which the morphological distinctiveness is the only accepted condition for registering a new species. For instance, reproductively isolated, cryptic species of *Aneura pinquis* will not be registered as long as morphological differences are found, even though their DNA fingerprints are completely different (Bączkiewicz et al. 2007). Likewise, since early 1980s sibling species of *Conocephalum conicum* (L.) have been recognized by means of isoenzymes and more recently DNA markers (Szweykowski et al. 1981; Sawicki et al. 2005). Nevertheless, they were elevated to the rank of taxonomic species with names *C. conicum* and *C. salebrosum* after distinct morphological traits had been identified (Szweykowski et al. 2005). At the opposite extreme, not everyone accepts objections to species status of morphologically different taxa, yet similar at the DNA level and not isolated reproductively. Even though, morphological differences are more related with varieties, ecotypes or environments. A nice empirical example of this sort involves classification of species within the genus *Lolium*. Apart from questionable status of *multiflorum* and *perenne* subspecies (Chapter 2-7), taxonomic position of the others is also subjected to a contentious debate over much of the twentieth century. How are the *Loliums* grouped depends both on morphological traits considered the most important and statistical or analytical methods employed. Traditionally, the species of the genus are recognised on the basis of such traits as plant height, leaf and spike morphology and many others (Chapter 1). Unfortunately, the majority of these characters are quantitative with the overlapping range of variation (Loos 1993a; Bennett 1997). It is not surprising because they are controlled by a huge number of QTLs distributed over the whole genome. Species have a mixture of positive and negative alleles at each QTL and none of them is fixed in a given taxa (Chapter 7). Although *L. temulentum* and *L. persicum* can be delimitated on the basis of floret number per a spikelet (Polok 2005) also this trait has a little value for taxonomy. At least six major QTLs are responsible for floret number and QTLs acting to increase the number of florets are equally distributed at least in *multiflorum* and *perenne* (Chapter 7). Serious difficulties are encountered as well when grain morphology is used for species classification. The seeds of out-pollinated taxa, *L. perenne* (ssp. *multiflorum* and ssp. *perenne*), *L. rigidum* and self-pollinated *L. loliaceum* are much the same.

A basic point for phylogenetic considerations is that evolution is a genetic process. Over time genome sequences are changing through different mutations (point, chromosomal, insertional etc.), that are further undergoing selection and eventually fixation. The extent of these changes reflects the evolutionary distinctiveness of populations, species and higher taxa. In addition, the changes are clocklike i.e., although the clock ticks at various speed across nucleotide positions within a codon, among different genes, among different classes of DNA within a genome and finally, among different genomes, the clock can be calibrated providing biogeographical, tectonic or any other past event is known (Savolainen and Chase

2003). From a practical point of view, this rate heterogeneity is highly beneficial by permitting for each kind of phylogenetic analyses from the population to higher taxa level. For example, the slowly evolving sequences are informative in reconstructing deep branches, while rapidly evolving sequences are especially useful in intraspecific analyses. The major strength of multi-character molecular approaches is that, unlike many morphological traits, the assumption of independence is probably valid. In any approach, molecular data are also well suited for constructing phylogenetic trees. With growing number of taxa surveyed at molecular level both phenetic and cladistic appraisals are important in resolving branch topology. With regard to *Lolium* a sad truth is that DNA assays have rarely been used to resolve evolutionary relationships within the genus. This is in spite of its economic importance, plenty of sophisticated analyses for *L. perenne* ssp. *perenne* and close relationships with cereals from which analytical procedures can be transferred easily. After all breeding strategies can often benefit from knowledge about evolutionary relationships of crops. A case in point involves management strategies aimed at conservation of turf characters in *perenne* and protection from undesired gene flow from *multiflorum* as well as introgression of advantageous genes from other members of the genus or closely related genera. The additional reason for phylogenetic studies is that they are guides to predicting risks of unwanted gene flow from improved transgenic grasses that are in the centre of attention of breeding companies (Yamada et al. 2005). Multiple molecular assays used in the preceding chapters have illustrated well how useful they can be in resolving species phylogenies within the genus *Lolium*. A lesson learnt from *L. perenne* is that its domestication resulting in differentiation into two subspecies, wild *perenne* and domesticated *multiflorum* has lasted only hundreds of years. Thereby the next arising question is whether *L. perenne* is separated from another allogamous species *L. rigidum* or they should be also joined into a single species as Bulińska-Radomska and Lester (1985) proposed? If not when did they diverge? What are the relationships within allo- and autogamous species? Do they really represent different evolutionary lines as it has been shown by katG markers (Polok 2005) or perhaps the whole genus should be treated as a single entity? Answering at least some of these questions was the goal of this part of research. With the vast molecular methods in hands and with data gathered during the studies on *multiflorum* and *perenne* the attempts were undertaken to reconstruct the phylogenetic relationships within the genus *Lolium*.

8.2. MATERIAL AND METHODS

All species belonging to the genus *Lolium* were analysed i.e., four autogamous species, *L. loliaceum*, *L. persicum*, *L. remotum*, and *L. temulentum* as well as two allogamous species, *L. perenne* with two subspecies *L. perenne* ssp. *perenne* and *L. perenne* ssp. *multiflorum*, and *L. rigidum*. In a case of *multiflorum* and *perenne* the bulk sample was made from all genotypes used in the genetic diversity studies. Moreover, the parental genotypes from genome mapping were used as a control to facilitate the identification of mapped markers. Endemic to the Canary Islands, *L. canariense* was not included owing to difficulties in obtaining sufficient number of viable seeds from gene banks. To facilitate tree rooting and help to establish character-state polarities *Festuca pratensis* and *Poa pratensis* were included as

outgroups. The seed sources of all species and the methodology of plant growing are listed in Annex 13.1. The identity of species from gene banks was checked using taxonomic keys. Bulks from 20 to 30 plants were analysed per a species.

Total DNA was isolated from individual plants according to the modified CTAB procedure (Annex 13.4). Several types of molecular markers were used in the studies. The analysis of organelle DNA included: restriction site polymorphism of chloroplast DNA (only *Hae*III was used because only this enzyme revealed polymorphism in *multiflorum* and *perenne*) of the non-coding region *psbC-trnS* in chloroplast genome (Annex 13.6), amplification polymorphism of intron between B and C exons of *nad1* gene (Annex 13.7), restriction polymorphism of the mitochondrial gene, LOLMTI originated from *L. perenne* ssp. *multiflorum* (Annex 13.7). Nuclear DNA was analysed by restriction site polymorphism of rDNA internal transcribed spacer using universal primers complementary to the conservative sequences near 18S and 5.8S rDNA borders as well as by primers complementary to *L. perenne* ssp. *perenne* spacer, LOLITS. Amplification polymorphism of intergenic spacer between two nuclear tRNA-Leu genes was included in the studies. Two sequences derived from Lol p I pollen allergen, LOLPISO5A, LOLPISO1A and a sequence derived from Lol p Ib pollen allergen, LOLLOPIB were analysed with respect to amplification polymorphism. A restriction map was made for LOLPISO5A using enzymes previously selected on the basis of restriction maps of sequences deposited in NCBI. The maps were created using NEB cutter program provided by New England Biolabs. Furthermore, the polymorphism of several low copy sequences was analysed. They included *L. temulentum* nuclear gene encoding chlorophyll binding protein type II (LTLHAB) and *L. perenne* genes encoding L-asparaginase (ASNL), thioredoxin (Trx) and glutamine synthetase (Gln2). Methodology of the analysis of all mentioned above sequences is given in Annex 13.14 while primer sequences in Annex 13.5. All random, genome scanning markers, RAPD, ISJ, AFLP and transposon-based SSAP markers that have been previously mapped on linkage map of *multiflorum* x *perenne* were included in phylogenetic analyses. In total 12 RAPD primers, 12 ISJ primers, six AFLP primers, two combinations of primers specific to the DNA transposon, *Tpo1* and two combinations specific to the *Lolcopia2* retrotransposon (Annex 13.08-13.12) were employed.

Genetic similarity was calculated based on the number of shared bands according to Nei and Li (1979) with further modifications introduced by Clark and Lanigan (1993) for RAPD data and Innan et al. (1999) for AFLP data. The resultant matrices were used in the cluster analysis. Two clustering methods were applied, the UPGMA that assumes equal evolutionary rates and the neighbour-joining (N-J) that allows for unequal rates. However, received trees were nearly identical, therefore only the results of UPGMA were presented owing to the possibility of using STATISTICA 7.1. Where necessary the principal component analysis was employed. The mean number of nucleotide substitutions for restriction sites was calculated according to Nei and Li (1979).

8.3. RESULTS

8.3.1. Diversity of organelle DNA in the genus *Lolium*

In total three different cpDNA haplotypes were identified using *Hae*III digestion (Figure 8.1). They did not group species in accordance with their taxonomy. The haplotype with three bands was typical of four taxa, *L. loliaceum*, *L. persicum* and two subspecies of *L. perenne* - *multiflorum* and *perenne.* The same haplotype was observed in *F. pratensis*. The other three *Lolium* species, *L. remotum*, *L. temulentum* and *L. rigidum* had a single fast band, probably resulted from the digestion into several very small bands not resolvable by agarose gels. The unique haplotype with five bands was observed in *P. pratensis.*

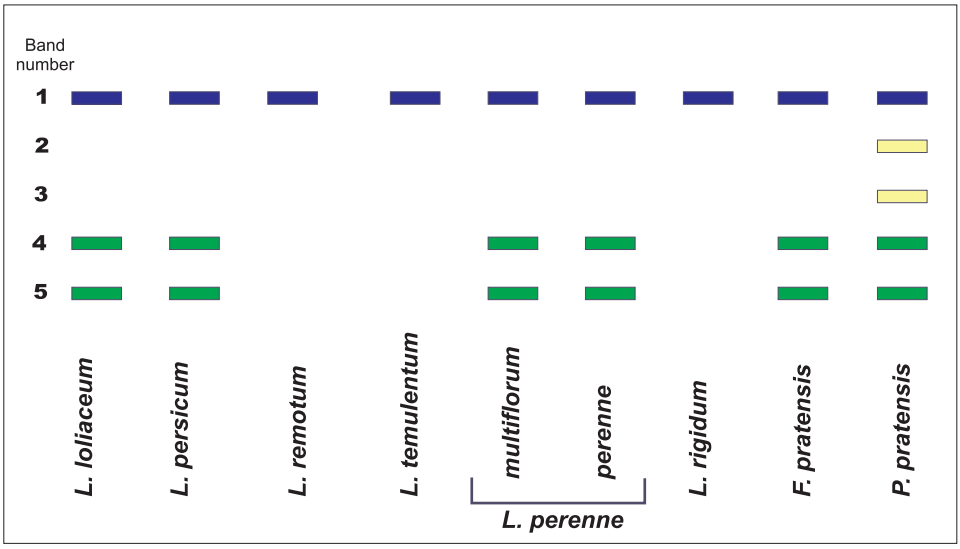


Figure 8.1. Haplotypes of cpDNA observed in species of the genus *Lolium*

The greater variation was observed among mtDNA haplotypes. They possessed from three to ten bands (Figure 8.2). Among them two bands were common to all studied species (band nº-1 and nº-5). The other two bands could be used as markers of two *Lolium* groups. The band Nº-3 was typical to all allogamous species. Its presence in *F. pratensis* confirmed close relationships between *Festuca* and *Lolium*. In contrast, its presence in *L. loliaceum* was somehow surprising because this species is normally classified in the autogamous group. On the other hand, three autogamous species, *L. persicum*, *L. remotum* and *L. temulentum* had clearly distinct haplotypes with a band Nº-9 as the most typical. This band was not observed in any allogamous species and *L. loliaceum*, but it could be found in *P. pratensis.*

When all bands observed for mtDNA were taken together, the clear picture was that three autogamous species, *L. persicum*, *L. remotum* and *L. temulentum* had the common haplotype, clearly distinct from the others taxa. The rest of studied species formed the second group with quite variable haplotypes from one species to another. Such diversity was also observed for different cultivars and ecotypes of *multiflorum* and *perenne* therefore, hap-

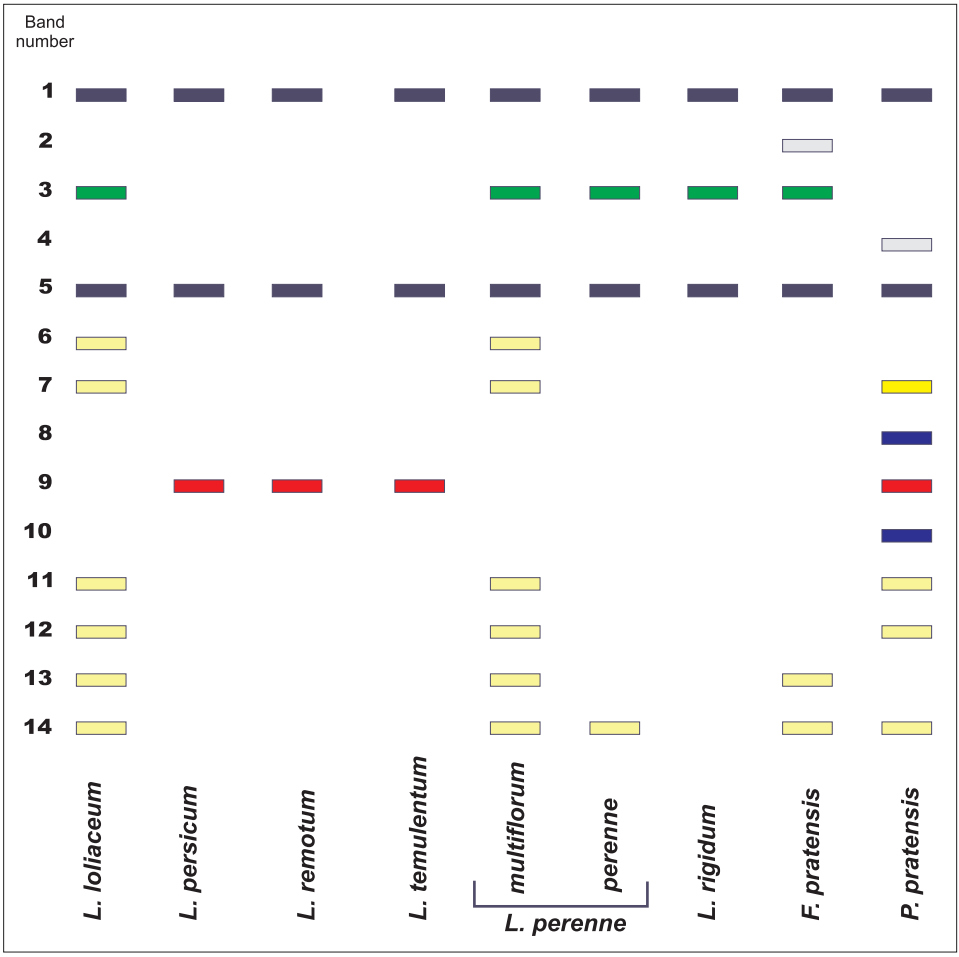


Figure 8.2. Haplotypes of mtDNA observed in species of the genus *Lolium*

lotypes could not be treated as species specific. They should rather be perceived as the typical feature of allogamous species. In spite of diverse haplotypes a certain degree of similarity could be found. The haplotypes of *L. perenne* ssp. *perenne* and *L. rigidum* were mostly the same with the only one additional band in *perenne*. Another subspecies, *multiflorum* apart from a common pattern typical of *perenne* and *L. rigidum* had additional five bands. All these bands were shared with *L. loliaceum* and moreover three with *P. pratensis*. Closely related with *Lolium*, *F. pratensis* had all bands but one typical of *Lolium*.

To study in more details relationships between *Lolium* species a mitochondrial gene derived from *L. perenne* ssp. *multiflorum* was amplified and after confirming that a single product was obtained, it was subjected to restriction analyses. Among seven *Lolium* taxa studied, only four gave a single band. These were all allogamous species (*multiflorum*, *perenne* and *L. rigidum*) in addition to autogamous *L. loliaceum*. Moreover, a single

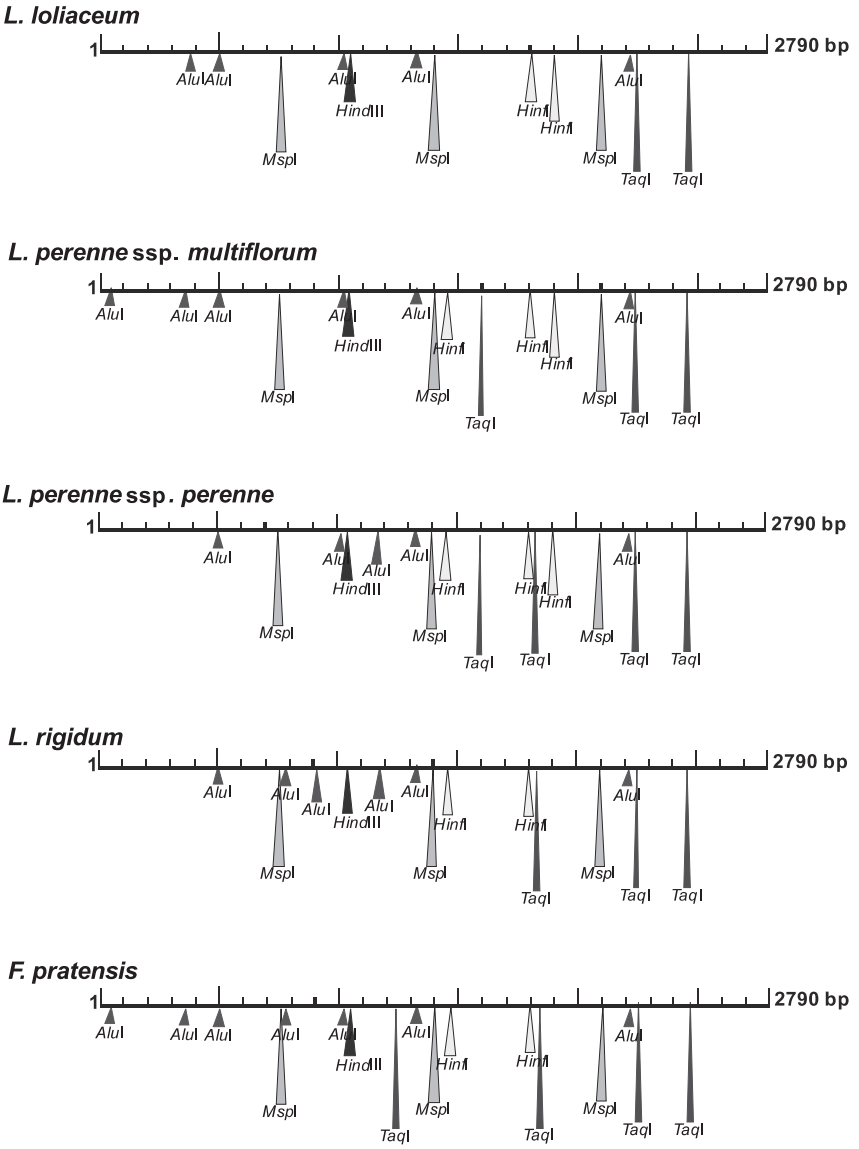


Figure 8.3. Restriction map of the mitochondrial gene, LOLMTI in four species of the genus *Lolium*

band was observed in *F. pratensis*. The rest of studied species gave a multi-band pattern that confirmed their distinctiveness. Restriction digestions were carried out only for species that amplified a single band. Restriction maps consisted from sites specific to five enzymes, *Alu*I, *Hinf*I, *Hind*III, *Msp*I and *Taq*I. The number of sites ranged from 13 in *L. loliaceum* to 17 in *F. pratensis*. The majority of sites were common for all or for the most of species (Figure 8.3). *F. pratensis* had only one unique *Taq*I site i.e., not observed in

any *Lolium* representatives. None restriction site was common for all *Lolium* species. Either a site was observed only in some of all analysed species or it was common to *Lolium* and *Festuca*.

The restriction map of *L. loliaceum* mitochondrial gene confirmed its close affinity to the group of out-pollinated taxa. It was the most alike *L. perenne* ssp. *multiflorum* that was reflected by only 0.039 nucleotide substitutions between both species in comparison with twofold more substitutions between *L. loliaceum* and *perenne* (Table 8.1). Additional reason why *L. loliaceum* was related with *multiflorum* the most closely was the unique *Alu*I site at 350 bp that was probably a sign of past introgression from *F. pratensis* to *multiflorum* and next to *L. loliaceum*. The lack of this site in *perenne* and *L. rigidum* seemed to confirm the evidence of introgression. Similar conclusion could be drawn from the lower number of nucleotide substitutions differentiating both *L. loliaceum* and *multiflorum* from *F. pratensis* in comparison with *L. rigidum*. The intermediate position of *L. perenne* ssp. *perenne* between *L. rigidum* and *multiflorum* was apparent from the distribution of *Taq*I sites. Two sites (at 1600 bp and 1820 bp) were present in *perenne*, the first was shared with *L. rigidum* whereas the second with *multiflorum*. It was also exemplified by the lowest mean number of nucleotide substitutions between *perenne* and *L. rigidum* from one side and *perenne* and *multiflorum* from the other.

Table 8.1. The mean number of nucleotide substitutions in the mitochondrial gene LOLMTI

	L. perenne ssp. *multiflorum*	*L. perenne ssp. perenne*	*L. rigidum*	*F. pratensis*
L. loliaceum	0.039	0.076	0.312	0.107
L. perenne ssp. multiflorum		0.033	0.055	0.050
L. perenne ssp. perenne			0.033	0.067
L. rigidum				0.045
F. pratensis				

8.3.2. Diversity of spacers in genes encoding 18S-26S rRNA and leucine tRNA

The amplification of ITS1 region using common conservative primers resulted in the single PCR product of the same size in all *Lolium* species in addition to *F. pratensis* and *P. pratensis*. The ITS region of all nine species had the same restriction sites with respect to *EcoR*V (data not shown) and *Taq*I (Figure 8.4), demonstrating inability to differentiate not only *Lolium* species but also the closely related taxa, *F. pratensis* and more surprisingly, *P. pratensis*. Eventually, minor differences were revealed by the digestion with *Hae*III (Figure 8.4). In that case all autogamous species but *L. loliaceum* were clearly distinct from allogamous ones. *F. pratensis* could easily be recognised, however the fingerprint of *P. pratensis* was very alike *Loliums*. The better resolution was obtained when primers complementary to the ITS region derived from *L. perenne* ssp. *perenne* - LOLITS were used for amplification. The difficulties with obtaining a single PCR product in *L. persicum* proved its distinctiveness from the other *Lolium* species. For the remaining taxa a specific PCR band was obtained allowing further restriction digestions. Similarly to ITS1, LOLITS was not digested by *EcoR*V and only minor differences could be observed in *Taq*I patterns. Nevertheless, the diges-

tion with *Hae*III gave quite surprising results. Two self-pollinating species, *L. remotum* and *L. temulentum* had identical patterns but clearly distinct from outbreeders (Figure 8.4). The unexpected findings involved the identity of their patterns with that of *F. pratensis*. Another point was that another self-pollinating species *L. loliaceum* had the pattern typical to out-pollinating taxa, *multiflorum*, *perenne* and *L. rigidum*. In summary, the ITS regions enabled to divide genus into auto- and allogamous species. In addition, *L. persicum* was clearly different from *L. remotum* and *L. temulentum*. Further distinction between these two species as well as between outbreeders was not possible. Moreover, the position of self-pollinating *L. loliaceum* was in disagreement with taxonomic position owing to high similarity to allogamous species.

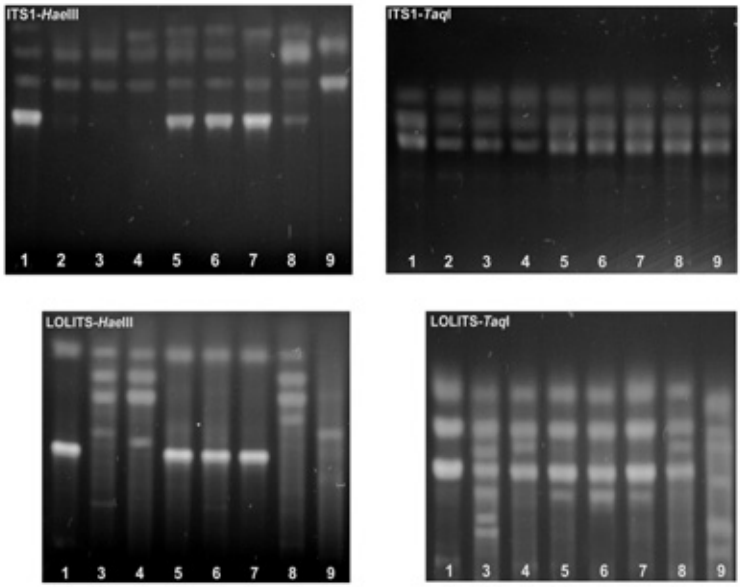


Figure 8.4. Restriction digestion of ITS region in nuclear rDNA in species of the genus *Lolium*
ITS1 – ITS1 fragment amplified with common conservative primers, LOLITS – ITS region amplified with primers complementary to *L. perenne* ITS (Accession L35517)
1. *L. loliaceum*, 2. *L. persicum*, 3. *L. remotum*, 4. *L. rigidum*, 5. *L. perenne* ssp. *multiflorum*, 6. *L. perenne* ssp. *perenne*, 7. *L. rigidum*, 8. *F. pratensis*, 9. *P. pratensis*

The placement of *L. loliaceum* together with out-pollinated taxa was confirmed by the analysis of intergenic spacer between genes encoding leucine tRNA (Figure 8.5). With this only exception, the t-RNA based tree grouped auto- and allogamous species quite well. Unsurprisingly, two subspecies of *L. perenne* were grouped together and then formed a cluster with *L. loliaceum*, that further joined with *F. pratensis*. The other cluster consisted from autogamous species with *L. remotum* and *L. temulentum* joining at the highest similarities and more distant *L. persicum*. As expected *P. pratensis* occupied the most external position. Strangely, but *L. rigidum* adopted slightly distant position from the rest *Loli-*

um species being the closest to *P. pratensis.* At the meantime the more central position of *F. pratensis* may imply its role as a common ancestor.

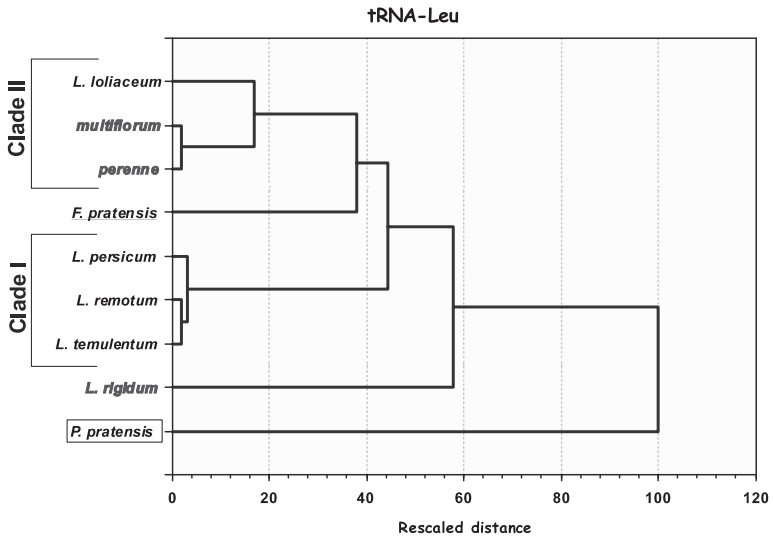


Figure 8.5. UPGMA grouping of the genus *Lolium* based on a spacer between leucine tRNA genes
multiflorum – *L. perenne* ssp. *multiflorum, perenne* – *L. perenne* ssp. *perenne*

8.3.3. Diversity of genes encoding pollen allergens and other low copy sequencesin the genus *Lolium*

The dendrograms based on pollen allergen genes and other low copy sequences demonstrated the ability to divide all *Lolium* species into two groups in general agreement with the mode of reproduction (Figure 8.6-8.7). Similarly to tRNA-based dendrograms, two clusters could be recognised. The first grouped self-pollinated *L. remotum*, *L. temulentum* and *L. persicum*. The second group was always formed from out-pollinated species, *L. rigidum* and *L. perenne* with two subspecies, *multiflorum* and *perenne*. Besides these firm groups some degree of variability in genetic divergence was found. The major difference between allergen- and other sequence-based dendrograms was the placement of *L. loliaceum* in proximity to *F. pratensis* and *P. pratensis* in the former. It again could be a reminiscence of the past introgression. Noteworthy, the allergen-based tree was the only one that grouped *L. remotum* closer to *L. persicum* than *L. temulentum.*

The nucleotide diversity of the LOLPISO5A gene was in general agreement with results of grouping and the taxonomic position of species, yet it revealed some surprising results. The difficulties in amplification of a gene derived from *perenne* in *L. rigidum* were unexpected, especially that a single band was easily obtainable in more distant species

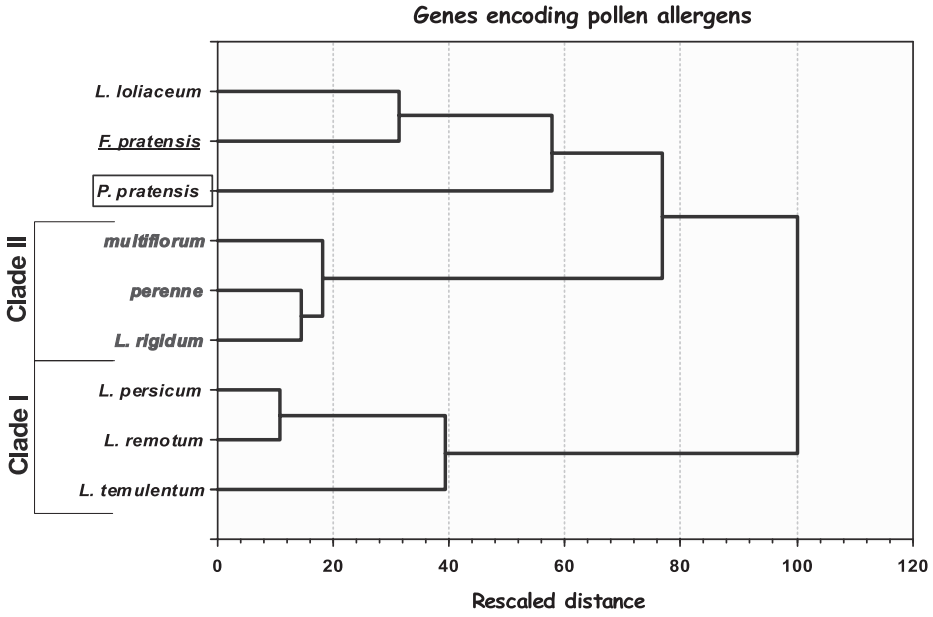


Figure 8.6. UPGMA grouping of the genus *Lolium* based on genes encoding pollen allergens
multiflorum — *L. perenne* ssp. *multiflorum*, *perenne* — *L. perenne* ssp. *perenne*

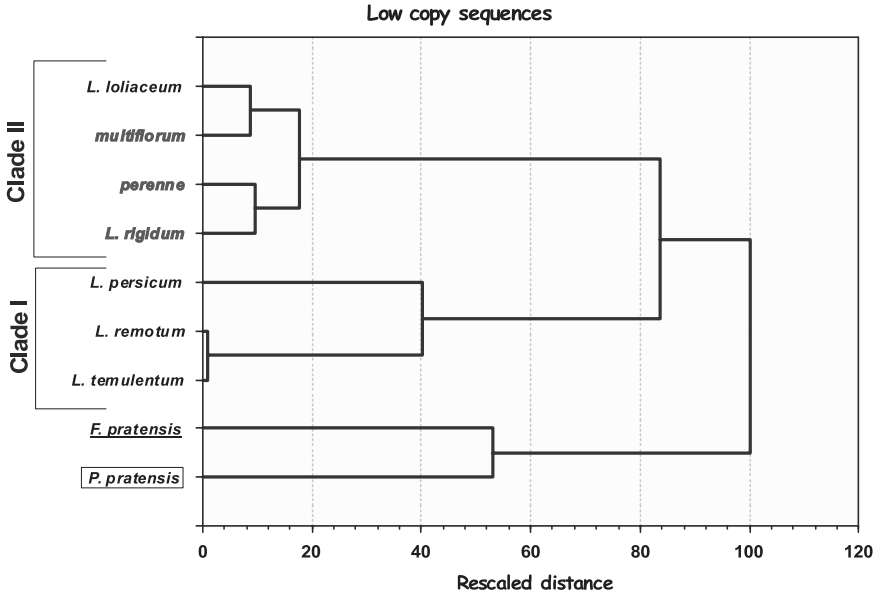


Figure 8.7. UPGMA grouping of the genus *Lolium* based on selected low copy sequences derived from *L. perenne* and *L. temulentum*
multiflorum — *L. perenne* ssp. *multiflorum*, *perenne* — *L. perenne* ssp. *perenne*

such as *L. remotum*, *L. temulentum* or *F. pratensis*. In total six taxa gave a single PCR product that was digested. Between 14 and 24 restriction sites specific to six enzymes (*Alu*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Taq*I) were identified. The magnitude of nucleotide divergence was the smallest between *L. remotum* and *L. temulentum* whereas the biggest values were observed between these both species and two subspecies of *L. perenne* (Table 8.2). Two species, *L. remotum* and *L. temulentum* were the most closely related as it could be concluded from shared restriction sites (Figure 8.8). It was plausible that 35% more restriction sites observed in two *L. perenne* subspecies and in *L. loliaceum* in comparison with *F. pratensis* resulted from mutations after a gene had been introgressed from *F. pratensis*. The lack of unique restriction sites for *F. pratensis* and their lower number seemed to confirm this view. Otherwise, it could suggest the ancestral position of *F. pratensis*. The autogamous species, *L. loliaceum* shared nearly all restriction sites with *multiflorum* with a single exception of *Msp*I at the position of 300 bp that was shared with *perenne*. This again confirmed the close affinity of *L. loliaceum* to allogamous species, mostly to *L. perenne* ssp. *multiflorum*.

Table 8.2. The mean number of nucleotide substitutions in the nuclear gene LOLPIS05A encoding pollen allergen Lol p I

	L. remotum	*L. temulentum*	*L. perenne ssp. multiflorum*	*L. perenne ssp. perenne*	*F. pratensis*
L. loliaceum	0.259	0.247	0.014	0.032	0.105
L. remotum		0.015	0.249	0.255	0.125
L. temulentum			0.237	0.243	0.195
L. perenne ssp. multiflorum				0.049	0.058
L. perenne ssp. perenne					0.119

8.3.4. Grouping based on insertional polymorphism (SSAP) and random markers (RAPD, ISJ, AFLP)

Dendrograms constructed based on insertional polymorphism (SSAP) and random molecular markers were highly congruent thus proved the tremendous utility of both approaches in phylogenetic studies. In both trees two clades were clearly visible, mostly in agreement with the mode of reproduction (Figure 8.9-8.10). The first clade consisted from three self-pollinated species, the second included three out-pollinated species and self-pollinated *L. loliaceum*. The most outer position was occupied, as expected, by *P. pratensis*. The placement of *F. pratensis* in the middle of dendrograms suggested its ancestral position in relation to the genus *Lolium*. Only minor differences were observed between both dendrograms and were related with *L. rigidum*. It was joined with *L. loliaceum* in the SSAP dendrogram whether random molecular markers placed it as an outer branch of the group of allogamous species, in close proximity to *F. pratensis*.

Principally, the trees based on SSAP and random markers were highly congruent with that based on tRNA and low copy sequences. However, the smallest branches were better supported on the SSAP and random markers' dendrograms. The trees based on single approaches generally tended to regroup species from one sequence to another. This observa-

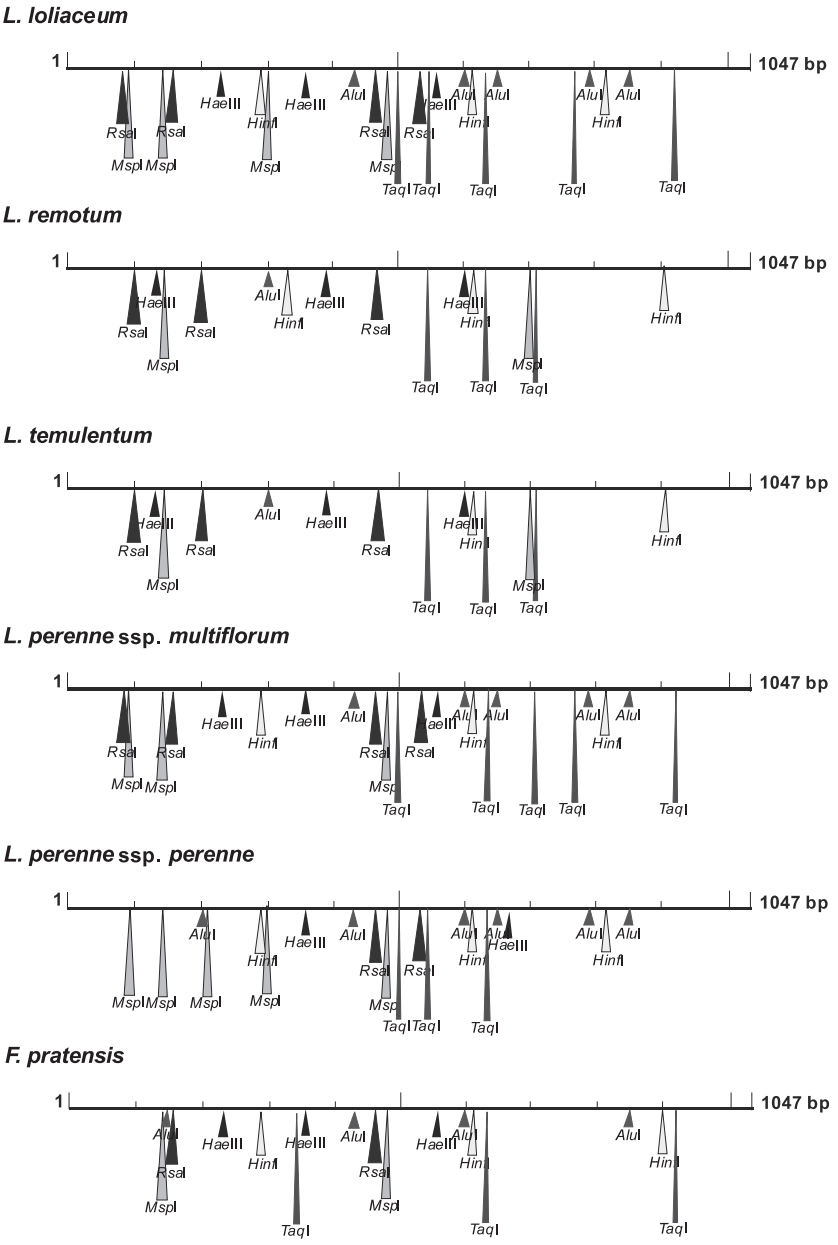


Figure 8.8. Restriction map of the pollen allergen gene LOLPISO5A derived from *L. perenne* ssp. *perenne* in species of the genus *Lolium*

tion supported the thesis that a single gene always produces some bias. Notwithstanding these minor differences, concordant phylogenetic signals recovered from all datasets enabled to combine them onto a single consensus tree that likely mirrored the phylogenesis of

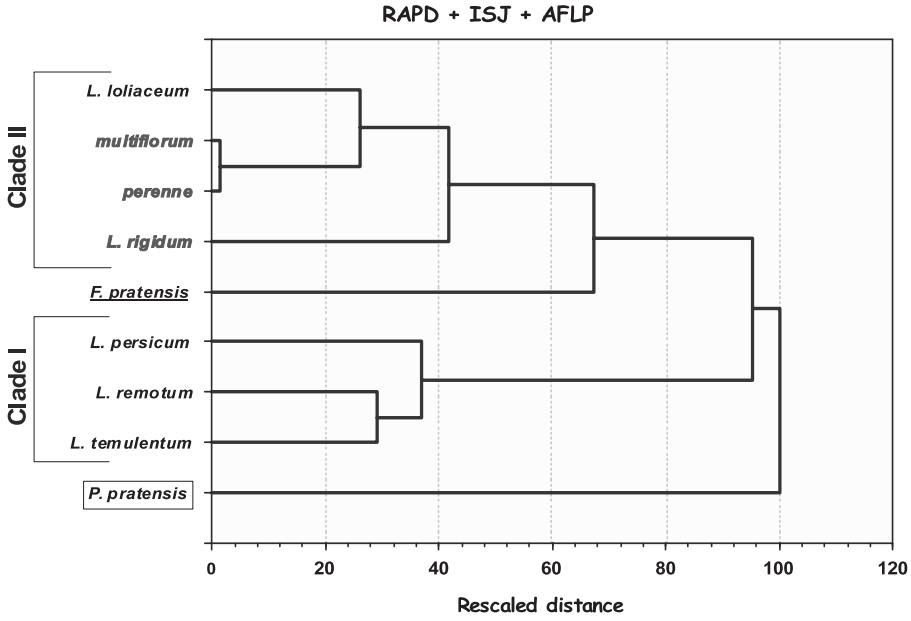


Figure 8.10. UPGMA grouping of the genus *Lolium* based on random molecular markers (RAPD, ISJ, AFLP)
multiflorum — L. perenne ssp. *multiflorum, perenne — L. perenne* ssp. *perenne*

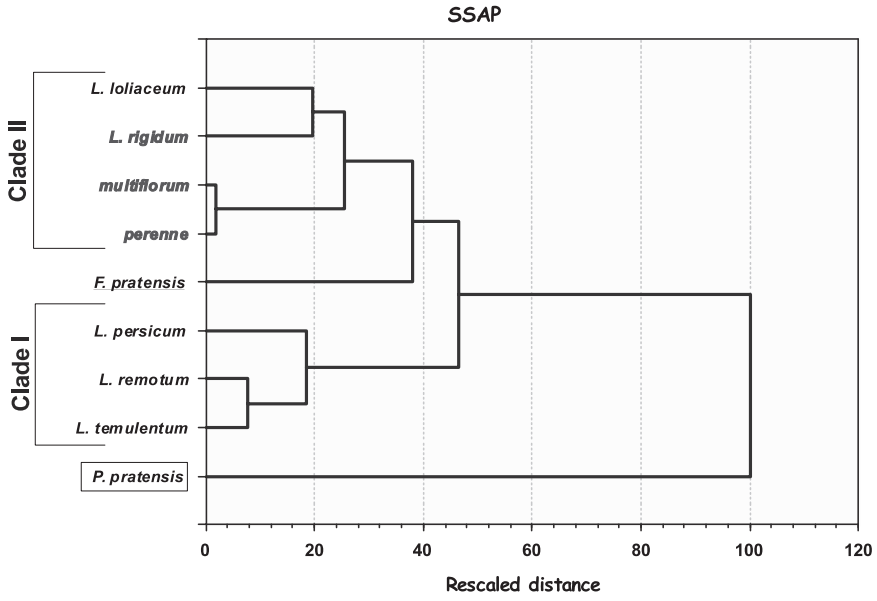


Figure 8.9. UPGMA grouping of the genus *Lolium* based on insertional polymorphism (SSAP)
multiflorum — L. perenne ssp. *multiflorum, perenne — L. perenne* ssp. *perenne*

the genus *Lolium* (Figure 8.11). It was also possible to calculate the values of Nei's genetic similarities summarized all over different genomic sequences (Table 8.3). It was likely that genetic similarities averaged for different sequences emphasized the genome diversities better because all types of sequences, from repetitive to low copy genes, from clustered to randomly distributed were sampled. Expectedly, the highest Nei's genetic similarity was between two *L. perenne* subspecies while the lowest between *Loliums* and *F. pratensis* or *P. pratensis.*

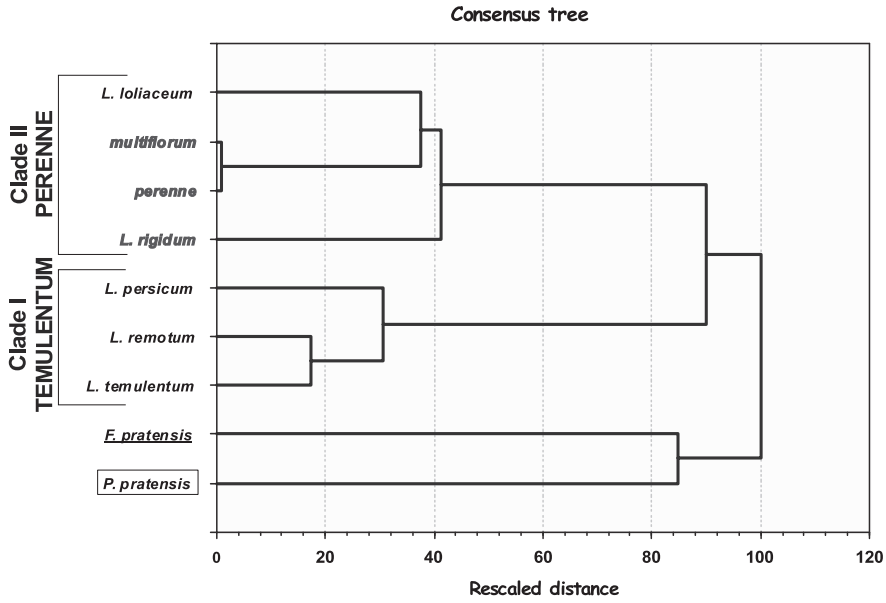


Figure 8.11. Consensus tree of the genus *Lolium* based on DNA data
multiflorum — *L. perenne* ssp. *multiflorum, perenne* — *L. perenne* ssp. *perenne*

The consensus tree gave the strong support for the existence of two clades that probably mirrored the evolutionary history of the genus. Unquestionable, three self-pollinated species, *L. temulentum*, *L. remotum* and *L. persicum* constituted the first clade further assigned as Temulentum clade. Within this group, *L. temulentum* and *L. remotum* were most alike, however the intermediate I value (0.682) suggested the relatively recent split between them. This clade was clearly distinct from the second cluster as well as from *F. pratensis*, joining with them at I=0.398 and 0.345, respectively. It indicated a significant degree of differentiation. The second clade further assigned as Perenne clade included four species, the most similar *L. perenne* ssp. *multiflorum* and *L. perenne* ssp. *perenne* (I=0.935) that joined with *L. loliaceum*. The similarity of 0.613-0.635 between *L. loliaceum* and *L. perenne* justified the recent origin of these taxa, but they appeared well separated. All three species formed a clade with *L. rigidum*, joining together at I=0.580 supporting the view that they are closely related, yet distinct species. The placement of *L. loliaceum* among outbreed-

ers appeared to register its different origin from the other self-pollinated species. Notable, as described above, the evolution of the second clade must have begun before *L. temulentum* and *L. remotum* from the first clade started to divide. *F. pratensis* and *P. pratensis* remained independent of these two clusters as the most distant species.

Table 8.3. Nei's genetic identity among species of the genus *Lolium* averaged for different molecular markers

	Autogamous species				Allogamous species			Outgroups	
					L. perenne				
	L. loliaceum	*L. persicum*	*L. remotum*	*L. temulentum*	*multiflorum*	*perenne*	*L. rigidum*	*F. pratensis*	*P. pratensis*
L. loliaceum	1.000	0.389	0.359	0.366	0.635	0.613	0.553	0.223	0.227
L. persicum		1.000	0.596	0.564	0.409	0.413	0.429	0.366	0.285
L. remotum			1.000	0.682	0.375	0.391	0.407	0.308	0.246
L. temulentum				1.000	0.396	0.414	0.424	0.355	0.294
L. perenne ssp. multiflorum					1.000	0.935	0.567	0.457	0.352
L. perenne ssp. perenne						1.000	0.570	0.458	0.341
L. rigidum							1.000	0.421	0.328
F. pratensis								1.000	0.302
P. pratensis									1.000

8.4. DISCUSSION

In *Lolium*, similarly to the other taxa, the enzyme electrophoresis was among the earliest molecular methods addressed to phylogeny reconstruction. These pioneering efforts have demonstrated that isozyme data correlate well with little morphological differentiation of the genus (Loos 1993b; Bennet et al. 2002). From all these comparisons an important conclusion has emerged that species within the genus *Lolium* have split only recently. Electrophoresis has also shown that the genus is differentiated into autogamous and allogamous species. The former group includes *L. loliaceum* (*L. subulatum*), *L. persicum*, *L. remotum* and *L. temulentum* while the latter *L. multiflorum*, *L. perenne* and *L. rigidum*. Own isozyme data do not support the division of *Loliums* into two groups. In the majority of enzymatic loci allelic variants common for all species are observed and relatively few species specific alleles are found thus demonstrating a common gene pool (Polok 2005). However, a concern about interpreting the evolutionary significance of the lack of enzyme variation in *Lolium* is that the resolution power of such markers may be too low. For example, available estimates of variation at enzymatic loci in closely related species *P. sylvestris*, *P. mugo* and P. *uliginosa* mirror only weakly their evolutionary history (Zielinski and Polok 2005; Polok et al. 2007). Using more powerful tools, namely non-coding cpDNA and mtDNA sequences, spacers in genes encoding rRNA and tRNA, several low copy sequences and plenty molecular markers, for the first time it is possible to shed light on evolutionary foot-

prints of species diversification within the genus *Lolium*. The current consensus tree has strong support in the congruent gene trees. It is based on the incredible number of 2 894 molecular markers representing different genes and repetitive sequences. Furthermore, the Mendelian inheritance was confirmed for many of them in previous mapping studies. Aside from 484 DNA markers mapped in the F_2 population of *multiflorum* and *perenne* during the present studies (Chapter 6), the Mendelian inheritance has been confirmed for the other 1 241 markers employing different crosses. The genetic map has also demonstrated that when all marker categories are taken together they are equally distributed within the genome and constitute the random sample of various DNA sequences.

When a tree is constructed from one gene, a reconstructed phylogeny represents a gene tree, which usually differs in topology from one gene to another. The tree of the genus *Lolium* based on genes controlling pollen allergens, groups *L. loliaceum* in a different way in comparison with the tree constructed from other low copy sequences. Some minor differences between tRNA, SSAP or random marker trees are found as well. However, the greatest disparities are observed between patterns revealed by cpDNA and mtDNA. It is not surprising because maternal (or any uniparental) inheritance of organelle DNA makes it especially prone to lineage sorting. This phenomenon is an inevitable consequence of differential reproduction. A simple case involves two individuals, each with different alleles at a given locus. One of these individuals does not leave any offspring whereas the other produces two individuals. Thus, the allele of the first individual is not represented in next generations but the allele of the second one is overrepresented. For relatively stable populations such lineages do not survive beyond 4N generations (N is the population size), yet they can create serious problems for phylogeny reconstruction at the species or population level (Avise 2004).

Concerted evolution is the second phenomenon that obscures seriously phylogenetic reconstructions based on moderately repetitive gene families. Concerted evolution is maintenance of a homogenous nucleotide sequence among the members of a gene family. It is emphasized by production of the same gene product from multiple loci. It results from gene conversion, the process in which a sequence from one locus is transferred unidirectionally to the other members of the gene family (Futuyma 2006). Because concerted evolution can be adaptive if large quantities of the product are needed, rDNA very often undergoes it and this can limit the utility of ITS approach in at least some taxa, including *Lolium*. For example, concerted ITS changes are assumed to occur even across different ITS arrays of monogenic Triticeae (Catalan et al. 2004). In *Lolium*, the lack of interspecific differences between ITS1 region and only minor differences revealed by LOLITS likely result from homogenizing the ITS region through concerted evolution. In comparison with the ITS region, tRNA spacers are more informative in terms of identifying species differences and building gene trees. First, they offer better assessment of phylogeny as it is exemplified by good species delimitation in the tree of the genus *Lolium*. Second, they are useful species specific markers as it is documented for liverworts from the genus *Pellia* (Fiedorow et al. 1998).

Notwithstanding the fact that no strongly conflicting placements of species are observed between different trees constructed for the genus *Lolium*, it should be admitted that each approach causes some bias. To exemplify, according to the tRNA tree *F. pratensis* occupies ancestral position in the middle of tree, genes encoding pollen allergens group it with *L. loliaceum* and *P. pratensis* while low copy sequences suggest its closest rela-

tionships with *P. pratensis*. Ultimately, its placement in the consensus tree together with *P. pratensis* and outside the genus *Lolium* is in agreement with current views on the evolution of *Lolium-Festuca* complex. Recognition of this fact leads to the important conclusion that only trees built on multiple genes or markers are likely to represent species trees. The ideal tree should have branch lengths proportional to evolutionary times. In practice, it is not easy to reconstruct such trees because evolutionary changes are subject to errors and selection (Nei and Kumar 2000). Therefore, it is important to use as many genes as possible to reconstruct species trees as it has been done in the current studies. Such choice of approaches guarantees the reliability of outcomes with minimal influence of reticulation and lineage sorting and constructed trees are likely to represent species trees. When supported with gene trees the species splitting patterns can be understood the best.

8.4.1. The progenitor of the genus *Lolium*

Owing to a small size there are no many potential progenitors of the genus *Lolium*. Protein studies suggest that ancestral forms have been most like the present *F. pratensis* and *L. perenne* (Bulińska-Radomska and Lester 1988). Similarly, Thomas (1981) postulated that speciation of the genus *Lolium* involved isolates of *L. perenne*. This thesis gains some support from the observations that most annual cereals evolved from perennial forms (Hu et al. 2003). Because *L. perenne* ssp. *perenne* is the only perennial grass within the genus, its ancestral position seemed plausible for early researchers. Nevertheless, the data presented here from genetic diversity studies, genome mapping and phylogenetic analyses as well as historical data (Beddows 1953; Casler and Duncan 2002) unambiguously exclude such possibility.

A counterproposal, more frequently expressed is that the Mediterranean basin is the centre of origin of the genus *Lolium*, and the common ancestor was most alike *L. rigidum* (Naylor and Rees 1958). The consensus tree based on all 2 894 molecular markers, and placement of *L. rigidum* in the middle among clades of self-pollinated and out-pollinated taxa, is in apparent agreement with the above view. If this scenario is correct, than the possible time of the common ancestor divergence from *Festuca*, estimated from cpDNA would be about 2 MYA and its divergence into species would be about 1 MYA (Charmet et al. 1997). Although this estimation is based on maternal lineages of the species, evolutionary rate is variable and it is a property of each gene (Senchina et al. 2003), the value of 2 MYA seems reasonable taking into account the results from the other Poaceae, for instance Oryzeae (Guo and Ge 2005). Hence, the cpDNA based value can be used to calibrate the molecular clock. On the other hand, the time of divergence can be calculated using $t=D/\alpha$, where α is the rate of nucleotide substitutions and D genetic distance. Taking 2×10^{-7} as a rough estimate of α (Nei and Kumar 2000), the divergence of *Lolium* from *Festuca* might have started about 2.5 MYA that is more or less in agreement with the cpDNA estimation.

The most serious obstacle is that the divergence of self pollinated species from the common ancestor should be calculated for 2.2 MYA. First, this timing is in great disagreement with that of Charmet et al. (1997). Second, it implies that either the diversification into species had started very early after separation from the common ancestor or the genus *Lolium* is not monophyletic. Monophyly of the genus *Lolium* has been questioned by Gaut et al. (2000) who

have indicated that the odd placement of one *F. pratensis*, greatly changes the tree. Moreover, the polyphyletic structure of *L. rigidum* accessions has been shown. Thereby, Gaut et al. (2000) concluded that the genus *Lolium* evolved from *Schedonorus* ancestor, of which *F. pratensis* is a representative. This theory would explain why *F. pratensis* occupies the position between groups of self-pollinated and out-pollinated species. The example includes the tRNA tree, SSAP tree and random marker tree. Notably, transposon-based markers that proved to be especially useful in phylogenetic studies in many plants, that elegantly resolve evolutionary relationships of *Pisum* (Vershinin et al. 2003), that are the only markers showing the early divergence of *multiflorum* from *perenne*, also indicate that the common ancestor is more similar to *F. pratensis*. The same picture emerges from a wealth of random markers. Hence, it is likely that the position of *F. pratensis* in the consensus tree is somehow disturbed by low copy gene markers, although from strictly statistical point of view it seems impossible. However, if the common ancestor had the closest affinity to *F. pratensis*, it is easier to explain the similar time when auto- and allogamous species began to diverge. It is also easier to explain the similarity between restriction patterns of the allergen encoding gene, LOLPISO5A in *F. pratensis* and both *L. remotum* and *L. temulentum.* This possibility is supported by the common cpDNA haplotypes between *L. persicum* and *F. pratensis* and completely different in L. *rigidum*. If autogamous species evolved from a common ancestor similar to *F. pratensis* then the haplotype of *L. persicum* originates from that ancestor. Under the above explanation another problem arises. *L. rigidum* has different structure of the LOLPISO5A gene as indicated by the lack of amplification products. Where does therefore, the gene in *multiflorum* and *perenne* originate from? A considerable body of data from breeding experiments has proved the presence of gene flows between different taxa of the genus *Lolium* and *Festuca* (Humphreys et al. 2003). Some *Festulolium* cultivars have been developed through crossing *multiflorum* with *F. pratensis* or with *F. arundinacea* and *perenne* with *F. pratensis* and have been introduced as novel temperate forage grasses in both Europe and the USA (Yamada et al. 2005). Hence, one of *L. perenne* subspecies might inherit the gene through introgression from *Festuca*. And because *perenne* and *multiflorum* are fully interfertile, the gene has become widespread in both subspecies. There are more and more data about increasing level of allergens in *L. perenne* (Sidoli et al. 1993) and the spontaneous as well as intended crosses with *Festuca* are the most likely reason. All the more the higher winter hardiness and disease resistance of *Festuca* make it an attractive component of crosses in many breeding programs. This hypothesis is reasonable in the light of the facts that natural *F. pratensis* x *L. perenne* hybrids are found throughout the British Isles and Northern Europe (Gaut et al. 2000).

To summarize, the more ancestral position of *F. pratensis* in comparison with *L. rigidum* in the majority of trees results from two different processes. Firstly, it is a reminiscence of past introgression accidents. Secondly, and more important, it mirrors the evolutionary relationships and its position as a common ancestor to both *Lolium* clades. Under this hypothesis, the divergence of the autogamous species from a common ancestor can be dated to 2.7 MYA and then the split between allogamous *Lolium* species and *Festuca* lineage can be postulated to 2.35 MYA. This theory inevitable entails the conclusion that *F. pratensis* should be classified within the genus *Lolium* as it has been proposed by Craven et al. (2005). Catalan et al. (2004) propose *Schedonorus* (broad leaved *Festuca*), of which

F. pratensis is the most prominent diploid representative, as the most common ancestor of the genus *Lolium*. Moreover, *Micropyropsis* is considered to be in between owing to a subracemose inflorescence, an intermediate trait between the paniculate of *Schedonorus* and the reduced spike of *Lolium*. What is more, the difficulties in the separation of the genus *Lolium* into two clades according to the mode of reproduction can be a further support of this hypothesis.

8.4.2. Division of the genus *Lolium* into two groups — auto- and allogamous species

The division of the genus *Lolium* into two sections in accordance with the mode of reproduction has long been postulated (Terrell 1968). The first section consists from strict annuals, self-pollinated species, *L. loliaceum*, *L. remotum*, *L. temulentum* and *L. persicum*, whereas out-pollinated *L. perenne* ssp. *multiflorum*, *L. perenne* ssp. *perenne* and *L. rigidum* belong to the second section (Jenkin 1959). The above classification has been confirmed in the majority of morphological and enzymatic studies (Loos 1993b; Charmet and Balfourier 1994; Bennet et al. 2002). Molecular approaches employed in the present work are in general agreement with the above picture. The genus can readily be divided into two clades corresponding to whether the plants are autogamous or allogamous.

The first clade consists of three species, *L. persicum*, *L. remotum* and *L. temulentum* that are self-fertile annuals, known only as weeds of cultivated crops. They possess unique mtDNA haplotypes and a little different ITS region as demonstrated by the restriction digestions of the LOLITS. Similarly to the other *Loliums* they have pollen allergen genes. Nevertheless, the mean number of nucleotide substitutions differentiating their allergen encoding genes from those observed in out-pollinated species is high and ranges from 0.237 to 0.255. The low average genetic similarity between autogamous and allogamous species (I=0.398) indicates a quite remote split between them. The second clade includes *L. perenne* ssp. *multiflorum*, *L. perenne* ssp. *perenne* and *L. rigidum* that are wind-pollinated annuals, biennials or perennials. They are easy to separate from all self-pollinated species due to different morphology and DNA fingerprints revealed by the majority of DNA markers.

Nonetheless, the position of the fourth autogamous species, *L. loliaceum* is contradictory with the current taxonomic classification according to which, the species should be included in a single clade with *L. persicum*, *L. temulentum* and *L. remotum*. Conversely, it consequently groups within out-pollinated taxa, in close proximity to *multiflorum* or *L. rigidum*, yet being distinct from them. Similarly, less nucleotides are different when restriction sites in *L. loliaceum* genes, both mitochondrial and allergen genes are compared with out-pollinated species than if such comparisons are made between *L. loliaceum* and self-pollinated taxa. The data from morpho-logical and isozyme analyses seem to confirm the close relationships of *L. loliaceum* with outbreeders. Morphologically, it is in somehow isolated position, more closely related to *L. rigidum* (Loos 1993a). Terrel (1968) interprets *L. loliaceum* as an offshoot of *L. rigidum*. Isozymes place it together with *multiflorum* and *L. rigidum* (Charmet and Balfourier 1994). The ability to separate *L. loliaceum* from the rest of inbreeding species based on isozymes confirms its distinctiveness. Note, the other autogamous species are unrecognisable at enzymatic level (Loos 1993b). Unfortunately, attempts to clarify the evolution of *L. loliaceum* based on DNA data have been hampered by the fact

that it has hardly been included in such analyses. Perhaps the underlying reason is the unclear position of this species that can spoil each dendrogram owing to disagreement with common classifications. For instance, the placement of *L. loliaceum* with *L. rigidum* in the ITS based dendrograms has led Gaut et al. (2000) to the conclusion that ITS does not provide consistent insights into the phylogenetic placement of this species. The first data that the origin of *L. loliaceum* may be different from the remaining autogamous species have come from AFLP studies (Polok et al. 2006). The placement of *L. loliaceum* together with *multiflorum*, *perenne* and *L. rigidum* demonstrates clearly, that it evolved within a group of out-pollinated species followed by several point mutations responsible for self-fertility. The results from the genus *Lycopersicon* indicates that the autogamy is controlled by at least five tightly linked genes controlling style length, stamen length and anther dehiscence. The clusters of genes associated with various aspects of transition from outcrossing to self-pollination probably exist in many plants and represent an ancient co-adapted gene complex controlling mating behaviour. A single mutation in *se2.1* gene on chromosome 1 of tomato results in more recessed stigmas and it is a major change accompanied the evolution from allogamous to autogamous species (Chen and Tanksley 2004).

Various molecular methods employed to assess phylogenetic relationships within the genus *Lolium* in the current studies unequivocally confirm the close affinity of *L. loliaceum* to out-pollinated species. In all dendrograms it consequently groups with *multiflorum*, *perenne* and *L. rigidum*. It has also similar cpDNA and mtDNA haplotypes, as well as the structure of the LOLMTI mitochondrial gene and the LOLPISO5A gene encoding pollen allergen. If *L. loliaceum* is included into the out-pollinated section than the genetic similarities between two clades drop down thereby, confirming that they are distinct lineages. For example, the genetic similarity between two “molecular” clades is twofold lower as estimated by AFLP and SSR and around 20-30% lower as indicated by the remaining molecular marker categories than for comparisons between autogamous vs. allogamous sections (Table 8.4).

Hence, the conclusion emerging from the current data is clear - the genus *Lolium* should be divided into two clades, the first consisted from *L. persicum*, *L. remotum* and *L. temulentum*, whereas the second from *L. loliaceum*, *L. perenne* ssp. *multiflorum*, *L. perenne* ssp. *perenne* and *L. rigidum*. These two clades as discussed earlier likely diverged independently from the common ancestor similar to the present *Schedonorus* genus. Under the above hypothesis, naming the two clades as groups of self-pollinated and out-pollinated species can not be held any longer for the reason that self-pollinated species are in both groups. It would be reasonably to assign both clades either after the progenitor or after the most common representative e.g., Temulentum clade for the first one, and Perenne clade for another. The time of divergence of the Temulentum clade from a common ancestor can be estimated as 2.5 MYA using molecular clock based on Charmet et al. (1997) calculations or as 2.7 MYA using $t=D/2\alpha$ transformation and 2×10^{-7} as the substitution rate. The Perenne clade split from *Schedonorus* (*F. pratensis*) about 2.35 MYA. Such scenario means that the ability to self-pollination arose independently in both clades as it has already been suggested using AFLP profiles (Polok et al. 2006).

Table 8.4. Comparison of genetic similarities between two clades of the genus *Lolium* based on DNA data

	SSR	RAPD	ISJ	AFLP	SSAP	B-SAP	
						IS6110	katG
Autogamous Section vs. *(L. loliaceum, L. persicum, L. remotum, L. temulentum)* **Allogamous Section** *(L. perenne* ssp. *multiflorum, L. perenne* ssp. *perenne, L. rigidum)*	0.48	0.71	0.67	0.73	0.70	0.67	0.64
Clade I (TEMULENTUM) vs. *(L. persicum, L. remotum, L. temulentum)* **Clade II (PERENNE)** *(L. loliaceum, L. perenne* ssp. *multiflorum, L. perenne* ssp. *perenne, L. rigidum)*	0.31	0.58	0.46	0.30	0.55	0.53	0.46

8.4.3. Phylogeny of two clades within the genus *Lolium*

When trying to clarify a sequence of events involved in the evolutionary development of the genus *Lolium*, like any other taxonomic group, it should be remembered that alleles are not only transferred vertically from parents to offspring, but hybridisation and introgression are quite frequent during a history of a species. Introgression is an important evolutionary process, by which species can acquire new genes enabled them to adapt to new environments or simple to overcome stresses. The evolutionary significance of introgression is well known but only recently it has been shown that hybridisation between distant species or even genera followed by introduction of genetic material is a regular process (Bachmann 2000). In California, the hybridisation between *Raphanus sativus* (cultivated radish) and *R. raphanistrum* (jointed charlock) is so extensive that both species have completely merged (Hancock 2005). Thus, there is no reason not to expect introgressions between closely related taxa such these from the genus *Lolium* and *Festuca*. The examples are well documented elsewhere and the introgression of pollen allergen from *F. pratensis* to *L. perenne* observed in the present work is just one of them. Notwithstanding the evolutionary significance of introgression, from a practical point of view it is very troublesome and undesired to everybody who would like to reconstruct phylogenesis. Ideally, it would be to have a single sequence applicable to each taxon at any level. For the sake of sustaining biodiversity it is only a dream. Each allele in a genome may represent different stories and as it has been documented here for the gene encoding pollen allergen, a given allele can originate from an introgression event in one species (*L. perenne*), whereas it is inherited from a common ancestor in another (*L. remotum* and *L. temulentum*). It is important therefore, to examine multiple sequences per species in phylogenetic studies. It should also be kept in mind that genomes predominantly consist of repetitive sequences, and among them retrotransposons are the most abundant. Hence, low copy genes or even members of multigene families that are liked so much in phylogenetic studies represent only a small piece of the whole diversity of the genome, and unfortunately a relatively conservative and unrepresentative piece. Thus, application of a sequence or a gene can be only a prelude to phylogenetic studies, enabling for preliminary diagnosis. With nearly 3 000 molecular markers based on different approaches and sampling various sequences from individual genes, through multigene families to highly repetitive transposons, this work can not be compared to any other. Such a wide choice of methodolo-

gies has been important in reconstructing the evolutionary history of the genus *Lolium* and enabled to elucidate the majority of unresolved problems. The phylogenetic relationships within the genus *Lolium* have been a subject of a debate for many years, however the majority of them have resulted from difficulties in the separation of *L. perenne* subspecies and furthermore from discrepancy in the classification of *L. loliaceum* between different studies. Once the solution to these problems has been achieved the phylogenetic tree of the genus *Lolium* becomes clear.

Clade I - TEMULENTUM

The clade I, named here as Temulentum, consists of three clearly distinct species, *L. persicum*, *L. remotum* and *L. temulentum*. The observation that *L. persicum* occupies the outer position to the *L. remotum* - *L. temulentum* cluster in all dendrograms with the only exception of pollen allergen genes, raises the possibility that *L. persicum* evolved from a common ancestor as the first. Charmet and Balfourier (1994) are in opinion that *L. persicum* is a derivative of the basic stock in Southwest Asia. The level of genetic similarities between *L. persicum* and other two taxa also is low (0.564-0.596) justifying its separation into a biological species. Furthermore, the existence of species boundaries is strongly supported by completely different fingerprints revealed by all marker categories. High degree of cytogenetic differentiation, extremely low pollen fertility (Senda et al. 2005) and seed set are further evidences of an intrinsic reproductive barrier.

The remaining members of the Temulentum clade, *L. remotum* and *L. temulentum* are very similar with the relatively high I value equal to 0.682 indicating a recent split between them. Numerically the value is typical of sibling or cryptic species i.e., species nearly identical morphologically but generally, readily separable by molecular markers (Zielinski and Polok 2005). Indeed, both species have some features of sibling taxa including only few definite observable differences between them (Bennet et al. 2002) coupled with preference to different habitats. *L. temulentum* occurs in wheat fields and *L. remotum* among flax. However, this is not to say that they are sibling species. In spite of some difficulties they are recognizable on the basis of seed morphology. This is to say that both species are very closely related, yet the postzygotic reproductive barrier exists as it is proved by crossing experiments. *L. remotum* x *L. temulentum* hybrids are perfectly normal throughout up to the point of pollen formation in the F_1 hybrids, when they fail (Jenkin 1959). According to Terrel (1968) both species originated from the same basic stock like *L. persicum* in Southwest Asia.

The timing of the sequences of evolutionary events among Temulentum clade is not easy due to conflicting information. Because the species are known only as weeds of crops they are thought to evolve in close association with primitive agriculture. Taking this fact at face value, Charmet and Balfourier (1994) estimated the most probable time of divergence between *L. remotum* and *L. temulentum* for 2000 years. Although this value seems compatible with the beginning of flax culture, it is not very plausible in the light of data from the other species or the timing of domestication processes in cereals. For instance, the basal split in the genus *Oryza* was about 8-10 MYA (Guo and Ge 2005), and allopolyploid cotton is estimated to have formed circa 1.5 MYA after the divergence of the diploid progenitor about 6.7 MYA (Senchina et al. 2003). According to cpDNA data, the differentiation of the genus *Lolium*

into species began about 1 MYA (Charmet et al. 1997). There is no doubt that the species within Temulentum clade should be older than 2000 years. Darnel, *L. temulentum* has been mentioned in the Bible, whose age is dated to 3500 years. When the divergence is estimated based on $t=D/2\alpha$ transformation, the respective values are as follow: the divergence of *L. persicum* from a common stock about 1.3 MYA, and the split of *L. remotum* and *L. temulentum* about 0.95 MYA. These values seems realistic in comparison both with 1 MY based on cpDNA (Charmet et al. 1997) and data from the other studies.

On the other hand, it is feasible that the Temulentum clade species observed nowadays resulted from a kind of unintended domestication during the development of agriculture. Domestication involves selection of seeds and modification of a number of traits including growth habit, plant architecture and the mode of reproduction. Growing in the field, between primitive crops, they might have been unrecognisable and harvested together with a crop. After some time first farmers might have learnt to recognise them as weeds and eliminate during a growth. Only individuals harvested with crops might have reproduced next season and thus only these plants were selected that were able first, not to be recognized, second, to produce seeds during a year. Crop mimicry i.e., the weed looks identical to the crop or weed seeds may be impossible to separate, has been particularly prevalent in grain cereals (Hancock 2005). Notably, self-pollination might have been an adaptation to growing with cereals and thus could have emerged late in the evolution of this group. The morphological similarity of *L. temulentum* seeds to barley or wheat or *L. remotum* seeds to flax seeds are the tangible evidences for the proposed scenario. *L. persicum* mimics the appearance of cereals so well that it can grow in cereal fields up it is to late. Therefore, it has become especially troublesome weed in the USA, where it has been introduced. As it is documented for such domesticated species as maize, barley and the others they do not differ from their progenitors making a single biological species. It is likely therefore, that today we are looking at domesticated forms of species belonging to the Temulentum clade. They differentiated about 0.95-1.3 MYA as estimated in the current studies and they were domesticated about 10 000 years ago together with the development of primitive agriculture.

Clade II - PERENNE

The clade Perenne consists of four taxa, allogamous *L. perenne* ssp. *multiflorum*, *L. perenne* ssp. *perenne, L. rigidum* and autogamous *L. loliaceum*. This clade seems to be less differentiated at least taking into account crossability between species. Therefore, the species delimitation has been a subject of a great contention for many years. The most controversies have been related with species status of *perenne* and *multiflorum*. The results presented throughout all chapters demonstrate clearly, that there is no any reproductive barrier between them and they can not be regarded as biological species. Furthermore, historical data (Beddows 1953) indicate that *multiflorum* is a domesticated form of *perenne* and the domestication process started in Italy before the twelfth century. The location of QTLs associated with the domestication syndrome is the other evidence confirming the historical records. The questions to be solved involve the diversification of *L. rigidum*, *L. loliaceum* and *L. perenne* from the common ancestor.

From the majority of dendrograms and additional considerations it is plausible that the earliest evidence of divergence within this clade is related to *L. rigidum*. Somehow different cpDNA and pollen allergen genes seem to support this view. The average I value equal to 0.563 between *L. rigidum* and the remaining species within the Perenne clade points at the presence of reproductive barriers. However, this prediction weakens considerably when data about full crossability between *L. rigidum* and the remaining species are taken into account (Loos 1993a; Bennet et al. 2002). Consequently, some authors classify *L. perenne* ssp. *multiflorum*, *L. perenne* ssp. *perenne* and *L. rigidum* as a single entity (Bulińska-Radomska and Lester 1985). On the other hand, there are hardly any documented data about the crossability of *L. rigidum* with the others and it remains to be established in experimental crosses. Indirect evidences come from hybrids between *L. loliaceum* x *L. rigidum* that are all male sterile (Jenkin 1959). Nevertheless, the present molecular data predict that such hybrids will be at least partially sterile. With regard to the likely time when *L. rigidum* diverged from the rest of the Perenne clade species, it can be estimated on about 1.4 MYA.

The divergence between *L. loliaceum* and *L. perenne* can be postulated to be about 1.2 MYA. It presumably followed several spontaneous mutations leading to self-pollination. In contrast to the self-pollination within the Temulentum clade, its birth in *L. loliaceum* was not connected with agriculture. Considering the value of genetic identity between *L. loliaceum* and *L. perenne* (I=0.624), they must have split only recently but the reproductive barriers must exist owing to low seed setting in hybrids between *L. perenne* ssp. *perenne* x *L. loliaceum*. A cautionary point should be made that low copy sequences group *L. loliaceum* more closely to *multiflorum* suggesting their common origin. The distribution of *Alu*I restriction sites in the LOLMTI mitochondrial gene and the LOLPISO5A gene encoding pollen allergen, *Rsa*I and *Taq*I sites in the latter also point at the closest affinity of *L. loliaceum* to *L. multiflorum*. On the other hand, such origin seems unlikely from at least two reasons. First, *multiflorum* and *perenne* have nearly the same genome and indeed, the former is a domesticated form of the latter. Under this hypothesis *L. loliaceum* would have been derived only recently from already selected forms as another domesticated form. However, in that case it should be much more similar to both *multiflorum* and *perenne* and the reproductive barrier would be rather excluded. Second, both mtDNA and allergens tend to cause bias in phylogenetic analyses. Therefore,

it is reasonable to think that *L. loliaceum* and *L. perenne* with both subspecies have the common ancestor such as *L. rigidum*. *L. loliaceum* could diverge earlier and that is why it occupies somehow intermediate position between *L. rigidum* and *L. perenne* entailing certain inconsistency between dendrograms. The placement of *L. loliaceum* with a sub-clade with *L. rigidum* in the SSAP tree is a strong support for the above hypothesis. Thus, the most plausible explanation of greatest similarity between *L. loliaceum* and *multiflorum* demonstrating by some molecular data is the hybridisation that has permitted gene introgression. Thus, the inclusion of the *multiflorum* sequence within *L. loliaceum* may be a reticulate event.

The beginning of diversification of *L. perenne* populations can be dated to about 167 000 years ago. It is worthy to note that it was a period of the Tertiary glaciations (Klimaszewski 1996) and the perennial type of growth habit might be a sign of past adaptation to harsh conditions. It would mean that perenniality is a relatively recent acquisition and that is why it is not so strong. From the present data and earlier considerations it can be postulated that *L. perenne* ssp. *perenne* evolved as a response to climatic changes and this adaptation enabled it to survive. During the interglacial period it became widespread throughout Europe and during the Quaternary Ice Ages its distribution became restricted to refugia. However, populations were not separated completely allowing gene flow due to existence of refugia also in the central part of Europe. In days of the Ice Ages it becomes widespread and extinct alternately. *L. perenne* ssp. *multiflorum* has arisen as populations more adapted to southern, warm climate but the gene flow disabled the speciation. Eventually, the unintended selection in the Middle-Ages resulted in the present domesticated form - *multiflorum*. Its mistaken elevation to a species rank by early taxonomists had led to much controversy that has been continued up to the present.

8.5. CONCLUSIONS (FIGURE 8.12)

1. The most probable common ancestor of the genus *Lolium* has the closest affinity to the *Schedonorus* subgenus, of which *F. pratensis* is the most prominent diploid representative.
2. The genus *Lolium* can be divided into two clades representing two different lineages that evolved independently from the common ancestor. The first clade (Temulentum) consists of *L. persicum*, *L. remotum* and *L. temulentum* and diverged 2.7 MYA. The second clade (Perenne) consists of *L. loliaceum*, *L. perenne* ssp. *multiflorum*, *L. perenne* ssp. *perenne* and *L. rigidum* and split from *Schedonorus* 2.35 MYA. Self-fertility arose independently in both clades.
3. Within the Temulentum clade, *L. persicum* was the first to diversify about 1.3 MYA and the separation between *L. remotum* and *L. temulentum* can be dated to 0.95 MYA. It is plausible that current forms of all species within that clade result from unintended domestication about 10 000 years ago.
4. Within the Perenne clade, *L. rigidum* split into a separate species about 1.4 MYA whereas *L. loliaceum* about 1.2 MYA. The latter originated through several point mutations that led to self-pollination.

5. The diversification of *L. perenne* populations started 167 000 years ago as a response to more harsh conditions during glaciations. In the Quaternary Ice Ages it was widespread and extinct alternately. However, its populations survived in refugia in south and central part of Europe. *L. perenne* ssp. *multiflorum* has adapted to more southern conditions. Through selection during domestication and elevation to a species rank the present *L. multiflorum* was created.
6. Spontaneous or artificial hybridisation between *L. perenne* and *F. pratensis* followed by introgression is responsible for incongruence in gene trees, and furthermore makes difficult interfering phylogeny from low copy sequences.

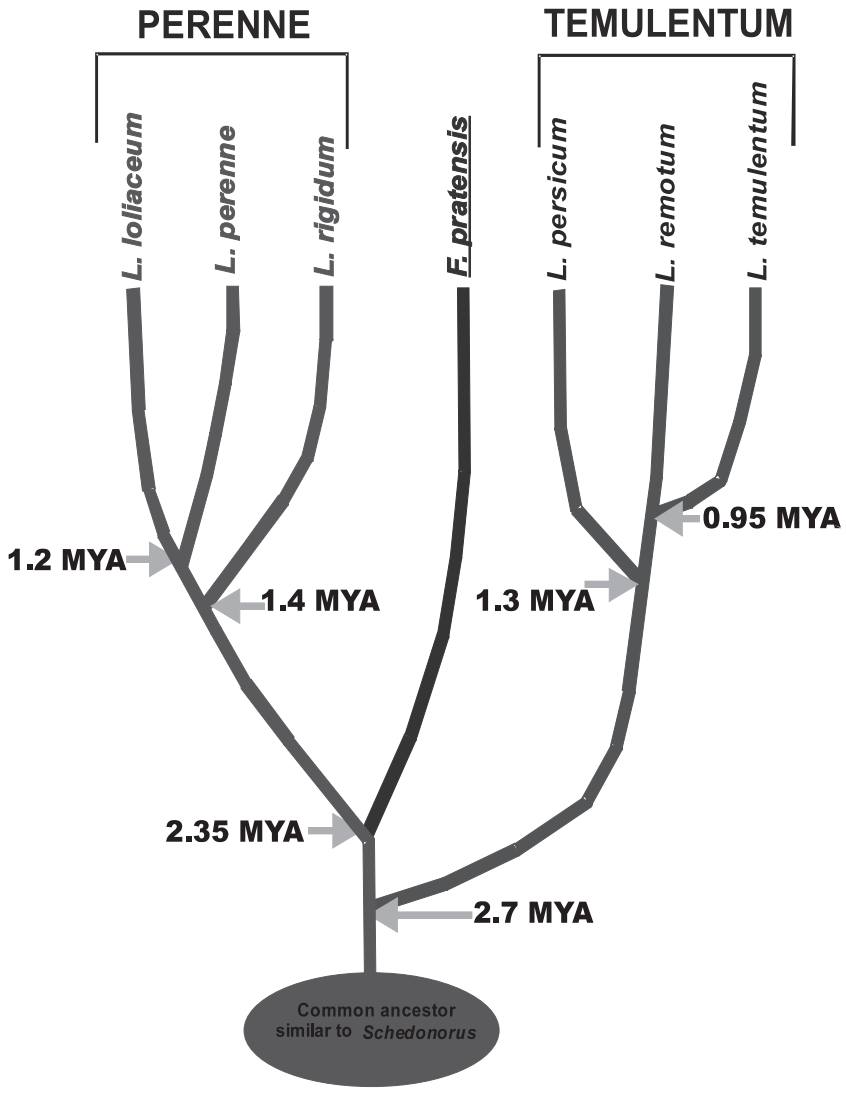


Figure 8.12. Hypothetical phylogeny of the genus *Lolium*

9. PHYLOGENETIC RELATIONSHIPS BETWEEN THE GENUS *LOLIUM* AND CEREALS

9.1. INTRODUCTION

Grasses have originated roughly 77 MYA and during evolution they have given enormous number of species widespread around the whole world. The family Poaceae includes 8 000-10 000 species that occur on all continents and are adapted to every terrestrial habitat covering over 20% of the earth's land surface (Kellog 1998; Gaut 2002). Huge morphological diversity and genome plasticity have enabled the first farmers to select plants with more useful characters. In that way they acted as new, previously unknown evolutionary forces entailing the development of crops that would never come into existence in nature and whose survival depends on human activities. This process commonly known as domestication has produced all major cereals (rice, maize, wheat, barley, oats) but also the minor grains (rye, teff) and other under-appreciated crops, for example Italian ryegrass, as it has been shown in the present studies. In many instances, the processes acting during domestication mirror those acting in nature, but due to strong selective pressure they are faster and more controlled. For this reason they are easier to follow and therefore they are a good model for understanding the nature of plant evolution. The disturbance of natural forest ecosystems and their transformation into cultivated fields has been a side effect of domestication. On the other hand it has prompted the evolution of new species adapted to these new environments in which grasses have been the most prominent component. Grasses are undergoing the adaptive radiation and with the current development of molecular methods we have an enormous possibility to track it.

From the perspective of the whole Poaceae family, the genus *Lolium* being only 2-2.7 million years old is one of the youngest. But it is also one of these genera, of which genomes are undergoing intense reshaping due to both natural and artificial processes. The youngest member of the genus *L. perenne* has probably started to diversify during glaciation periods about 160 000 years ago. As an answer to more harsh conditions, annual forms evolved into perennials. However, climatic changes during the Tertiary and Quaternary with glaciations and inter-glaciation periods caused that genes responsible for perenniality and annual growth persist in populations giving the opportunity for further diversification into more southern annual types and more northern, perennial types. On the other hand breeding activities can inevitably entail the selection of new forms as it has been exemplified by the creation of *multiflorum* - the domesticated form of *perenne*. When natural changes are coupled with artificial selection as it is in *Lolium* the evolutionary processes can significantly be fastened. The

current data exemplify how the nature of evolutionary changes can be traced using molecular appraisals. The documented examples of genome rearrangements responsible for the transition from wild to domesticated forms are also more and more frequent and involved such crops as maize, rice, sorghum and many others. However, all they document past events. The genus *Lolium* and especially *L. perenne* is unusual due to the fact that it is in a half way, it is diversifying both through the adaptation to more southern environments and domestication processes, but still pending the birth of species boundaries. It is a kind of a model for tracing "the evolution in action" through the use of vast molecular techniques available.

The results presented in the preceding chapters shed some light on the evolutionary history of the genus and such as they are dealing with the past. Another challenge is to use this knowledge to understand the nature of undergoing evolutionary processes driving by intensive breeding and changing environment. It would have a tremendous effect on predicting the potential influence of intergeneric hybrid cultivars or transgenic plants and finally help in protecting biodiversity. Close relationships of the genus *Lolium* with cereals or more generally with "Core Pooids" (Aveneae, Bromeae, Poeae, Triticeae) is an additional advantage because evolutionary considerations can draw extensively on the knowledge and methods developed for more studied species. Therefore, the present part intended to summarize the knowledge about the position of the genus *Lolium* within the Poaceae family and its relationships with cereals based on own results, with the aim of using it as a foundation for further research on the mechanisms of evolution in plants.

9.2. MATERIAL AND METHODS

In addition to seven species from the genus *Lolium*, described previously (Chapter 8, Annex 13.1) a set of representatives of cereals' species was used. It involved members of "Core Poids", *H. vulgare*, *T. aestivum*, *S. cereale* (Triticeae) and artificial allopolyploid derived from these two species - *Triticale*, and two oat species, *A. sativa* and *A. strigosa* (Aveneae). Moreover, *A. thaliana* was used as outgroup.

Phylogenetic trees were constructed on the basis of cereal sequence tagged sites (STS) previously used in mapping studies and selected from Taylor et al. (2001). Shortly, primers were derived from genes encoding asparagine synthetase (AS1), L-asparaginase (ASN), and HS1 protein of *H. vulgare*. In addition primers complementary to RFLP probes from *H. vulgare* (BCD450) and *A. sativa* (CDO504 and CDO1508) were used. PCR conditions were optimised during mapping studies so that to amplify a single band in *L. perenne* ssp. *multiflorum* and *L. perenne* ssp. *perenne*, and these conditions were used for species comparisons. Optimised PCR conditions are given in Annex 13.14. Primer sequences are given in Annex 13.5. A single band was amplified by primers derived from *H. vulgare* asparagine synthetase (AS1) and *A. sativa* RFLP probe CDO1508. Primers derived from *H. vulgare* RFLP probe BCD450 and *A. sativa* probe CD0504 revealed two strong reproducible bands whereas for ASN and HS1 only multi-band pattern was observed. In these cases the optimisation was done to receive reproducible amplification. At the next step DNA of all species was amplified at PCR conditions specific to *L. perenne*. If a single band was obtained, PCR products were subjected to restriction digestion. If two bands were obtained, the strongest band

was eluted from the gel, re-amplified and PCR products were digested. In total six restriction enzymes were used, *Alu*I, *BamH*I, *EcoR*I, *Hind*III, *Rsa*I and *Taq*I. In a case of multiple bands the polymorphism was assessed with respect to amplification products.

The utility of B-SAP for phylogenetic studies was checked using primers derived from *M. tuberculosis* gene encoding catalase-peroxidase, *KatG* and composing of 4 801 bp. Twelve pairs of katG primers were designed for every 280 bp fragments according to Zielinski and Polok (2005). The strategy of primer designing and PCR conditions are described in Annex 13.13.

The consensus tree was based on 2894 markers including RAPD, ISJ, AFLP, SSAP, SSR markers in addition to B-SAP markers derived from *KatG* gene, IS6110, *rpo* and *pol* from *M. tuberculosis*, markers derived from low-copy sequences of *Lolium* (LOLPISO5A, LOLPISO1A, LOLOPIB, LTLHAB, ASNL, Trx, Gln2), *H. vulgare* (ASN, AS1, HS1, BCD450) and *A. sativa* (CDO504, CDO1508).

The similarity was estimated on the basis of shared amplification products or restriction fragments according to Nei and Li (1979). The UPGMA and squared Euclidean distance were used for clustering.

Data from genetic mapping were compared with barley and oat maps. In particular, the location of enzymatic sequences and the abundance of transposons were compared.

9.3. RESULTS AND DISCUSSION

9.3.1. Phylogenetic relationships between the genus *Lolium* and representativesof "Core Pooids"

PCR-mediated gene sequencing for phylogenetic studies has increased explosively in recent years. Sequence data provide high-resolution picture of molecular diversity but sacrifice genetic information from many loci. Typically, sequence analysis involves a single gene in one or two individuals per a species. In that sense the DNA sequence data permit recovery of genetic information at less detailed level than some other kind of molecular markers. In *Lolium* the phylogenetic relationships are better resolved using different categories of molecular markers than analysing the LOLMTI mitochondrial sequence or the LOLPISO5A gene encoding *L. perenne* pollen allergen. Even the analysis of ITS in rDNA gives worse resolution than a wealth of molecular markers. A general view emerging from phylogenetic studies based on one or a few sequences is that they usually support for the higher taxa level but the support for the species level is very low. Furthermore, gene sequences are far more prone to phylogenetic noise than molecular markers because a nucleotide has only four alternative states. A practical income from analyses of DNA sequences is that a position of a given species tends to change depending on a gene used.

To use as many markers as possible to interfere the phylogenetic relationships seems to be the most fruitful approach, especially if many different marker categories are available and the band identity is confirmed in mapping studies. The data from the genus *Lolium* (Chapter 8) as well as the current tree of Pooideae subfamily demonstrate the high reliability of such strategy. The majority of branches were resolved well and the species relationships

could easily be determined (Figure 9.1). The unrelated *A. thaliana* was classified as the outgroup taxon. The remaining clades mirrored perfectly the evolutionary relationships between the species. Expectedly, the genus *Lolium* was placed within the clade Poeae in close proximity to *F. pratensis* and *P. pratensis*. Two sub-clusters corresponded to the clade I (Temulentum) and clade II (Perenne) as it was identified in the previous analyses (Chapter 8). The Poeae clade joined with the cluster grouping species belonging to the Triticeae and Aveneae clades. The former consisted of *T. aestivum*, *S. cereale* and *Triticale* that were joined with *H. vulgare*, whereas the latter from *A. sativa* and *A. strigosa*. This grouping is in accordance with the known phylogenetic relationships of cereals and their possible time of divergence. The Triticeae tribe diverged from Aveneae 25 MYA, *T. aestivum* and *H. vulgare* diverged 13 MYA (Gaut 2002). It is worthy to note that the intermediate position of *Triticale* was in agreement with its origin as a hybrid between *T. aestivum* and *S. cereale*. The closer position to wheat reflected its greater share in the constitution of the *Triticale* genome.

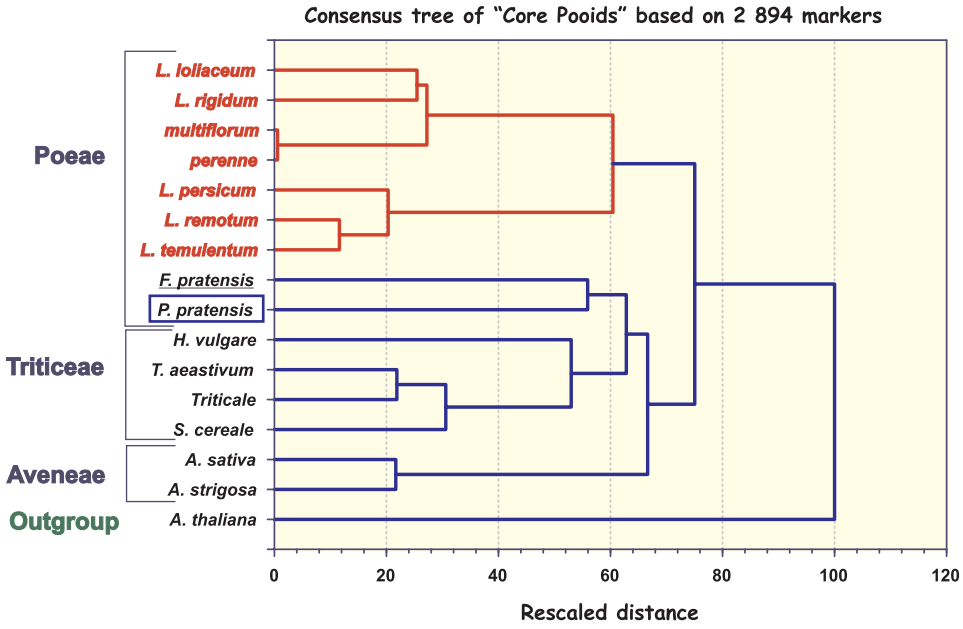


Figure 9.1. UPGMA grouping of „Core Pooids" based on molecular markers
multiflorum — *L. perenne* ssp. *multiflorum, perenne* — *L. perenne* ssp. *perenne*

In the present tree the Triticeae and Aveneae tribes were clustered forming a bigger clade while *Lolium* constituted another cluster. Such grouping indicates the closer relationships between Triticeae and Aveneae than between both of them and *Lolium*. It is not surprising, although some phylogenetic analyses often place Aveneae closer to Poeae (Kellog 1998; Mathews et al. 2000). This discrepancy can be a result of sampling. The tree resolution depends not only on molecular assay but, more important on species sampling. However, the aim of the present studies was to establish the relationships between the genus *Lolium*

and cereals, not to study the evolution of the whole Pooideae sub-family. Thereby no wild species were sampled.

Similarly, somehow surprising is the closer affinity of *F. pratensis* and *P. pratensis* to Triticeae than to *Lolium*. *F. pratensis* and *P. pratensis* formed a small cluster that further joined with cereals. On the other hand, the central placement of both species may reflect the ancestral position to *Lolium* that is in agreement with previous data. The ancestral position of *F. pratensis* is strongly supported by the Principal Component Analysis. In the PCA scatterplot *F. pratensis* was placed somewhere in the middle between two *Lolium* clades and *P. pratensis* (Figure 9.2). The last mentioned species was grouped with cereals. Noteworthy, the placement of *L. loliaceum* between *L. rigidum* and *L. perenne* provides further credence to earlier conclusion that *L. loliaceum* and *L. perenne* evolved from a common ancestor that had the closest affinity to *L. rigidum*.

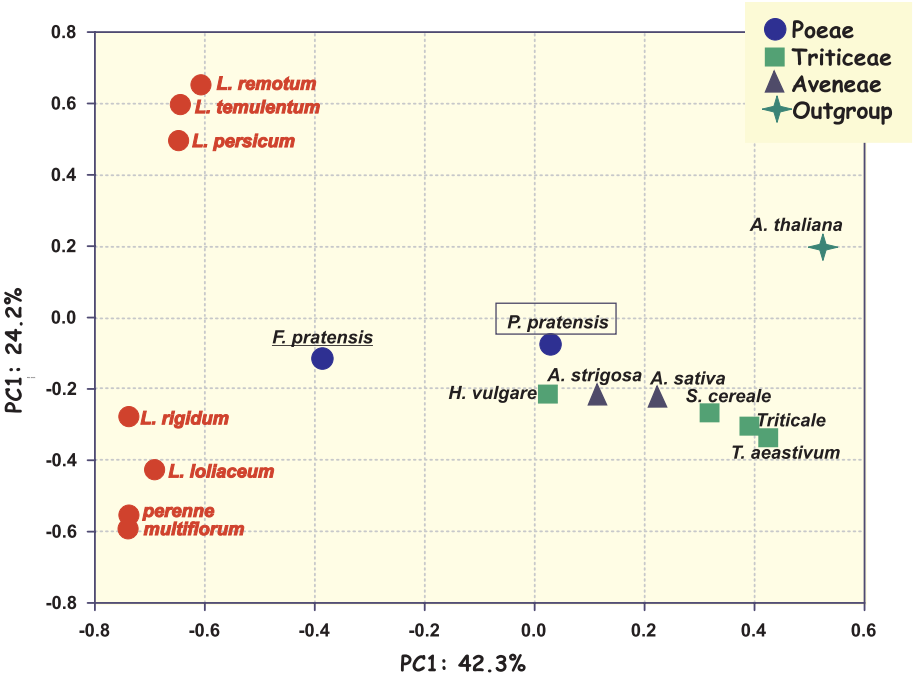


Figure 9.2. Principal component analysis of „Core Pooids" based on 2894 molecular markers
multiflorum – *L. perenne* ssp. *multiflorum*, *perenne* – *L. perenne* ssp. *perenne*

Not unexpectedly, the species delimitation is worse in the tree derived from cereal sequence tagged sites (STS). The most surprising was the dispersion of two *Lolium* clades into separate clusters (Figure 9.3). The Perenne clade formed a cluster with the Aveneae and Triticeae tribes whereas the Temulentum clade was joined with *F. pratensis* and *P. pratensis*. There may be plenty of biological and purely statistical explanations why the Perenne clade clusters with *A. sativa* and *A. strigosa*. However, two possibilities are the

most plausible. First, grouping is associated with the nature of STS used. If they encode proteins important in metabolic pathways ensuring the bodily functions, they are likely to be conservative through millions of years during evolution. On the other hand, in the light of difficulties in obtaining a specific PCR product, this explanation is not very convincing. Another class of arguments comes from the other trees. The close relationships between Aveneae and *L. perenne* are observed in the tree based on chloroplast *ndhF* gene sequences (Catalan et al. 1997). *L. perenne* forms a cluster with *Festuca arundinacea*, *F. rubra* and *Dactylis glomerata* and then it joins with the cluster of *Avena fatua*, *A. sativa* and *Arrhenatherum elatius*. Such relationships are observed neither in the tree based on phythochrome B (Mathews et al. 2000) nor in the tree derived from genome size data (Kellog 1998). When cpDNA and nuclear DNA show the same, but disturbed pattern, it is strong argument for the past introgression. Although, it looks unlikely that hybridisation coupled with introgression could take place between such distant taxa as oats and ryegrasses, yet it is possible. Hybridisation and introgression between crops and wide relatives has been documented for nearly 30 species, including maize, wheat, barley, oats and many others (Jarvis and Hodkin 1999). A few natural introgressive hybrids between herbicide resistant wheat and *Aegilops cylindrica* and between wheat and rye have been observed in North America (Hedge and Waines 2004). Nevertheless, the most prominent example comes from *Hordeum marinum* (sea barley) and *T. aestivum*. In northern Europe sea barley occasionally grows in direct proximity to wheat fields and has been described as being not strictly autogamous. In one individual, that morphologically was typical of *H. marinum*, numerous species-specific DNA markers of wheat were amplified demonstrating previous hybridisation (Guadagnuolo et al. 2001). Thereby, it is highly probably that the past introgression is the reason for grouping *Lolium* with Aveneae both in the current tree based on cereal genes and on the cpDNA tree of Catalan et al. (1997). Presumably, the other incongruence in the cereal genes-derived tree is also a reminiscence of the past introgression. *L. loliaceum* that belongs to the Perenne clade was grouped with *F. pratensis*. The same pattern has been observed for genes encoding pollen allergens. The restriction analysis has provided the clear evidence of the past introgression (Chapter 8). All these examples demonstrate clearly that dendrograms based on a single sequence or even a group of sequences but of the same type, should be treated with a great caution when used for the reconstruction of phylogeny.

When used for the reconstruction of phylogeny, the B-SAP-katG markers derived from catalase-peroxidase gene of *M. tuberculosis* present themselves in the best possible light. Not only the resolution of the dendrogram is almost perfect but also the relationships between species are in agreement with known evolutionary history. Two *Lolium* clades were clustered according to earlier reconstructed phylogeny of this genus (Chapter 8), but more important, *F. pratensis* occupied the ancestral position between them (Figure 9.4). It confirms the previous view that two groups of *Lolium* species evolved independently from a common ancestor of the *F. pratensis* type. Notable, the reliability of this marker system was demonstrated by forming a cluster consisted of *T. aestivum* and *Triticale*, which joined with *S. cereale*. The presence of a cluster that grouped *P. pratensis* and *A. thaliana* also seems logic. The former species is rather distantly related with both *Lolium* and cereals. According to Charmet et al. (1997) the *Festuca-Lolium* complex diverged from *Poa*

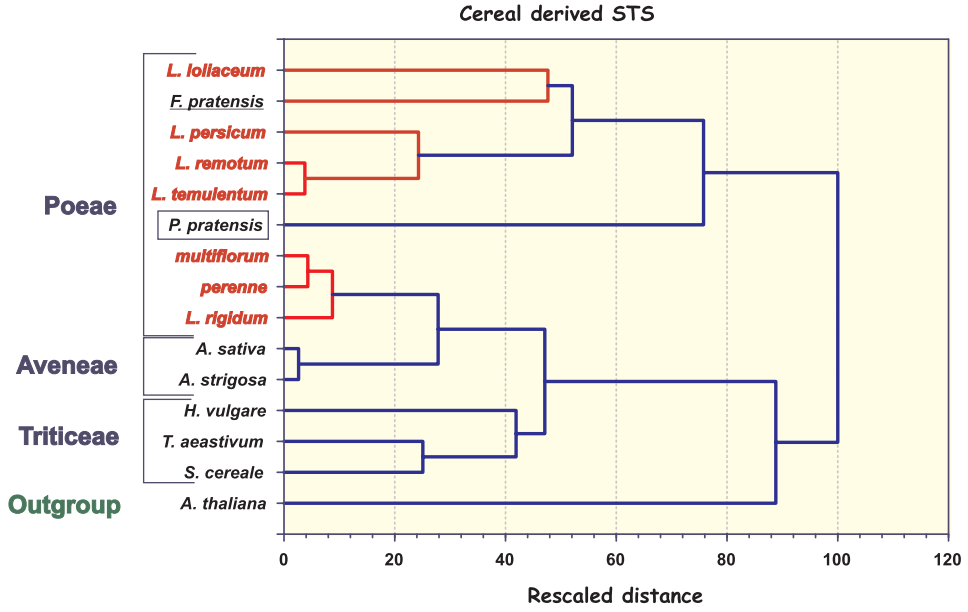


Figure 9.3. UPGMA grouping of „Core Pooids" based on STS derived from cereals
multiflorum — L. perenne ssp. *multiflorum, perenne — L. perenne* ssp. *perenne*

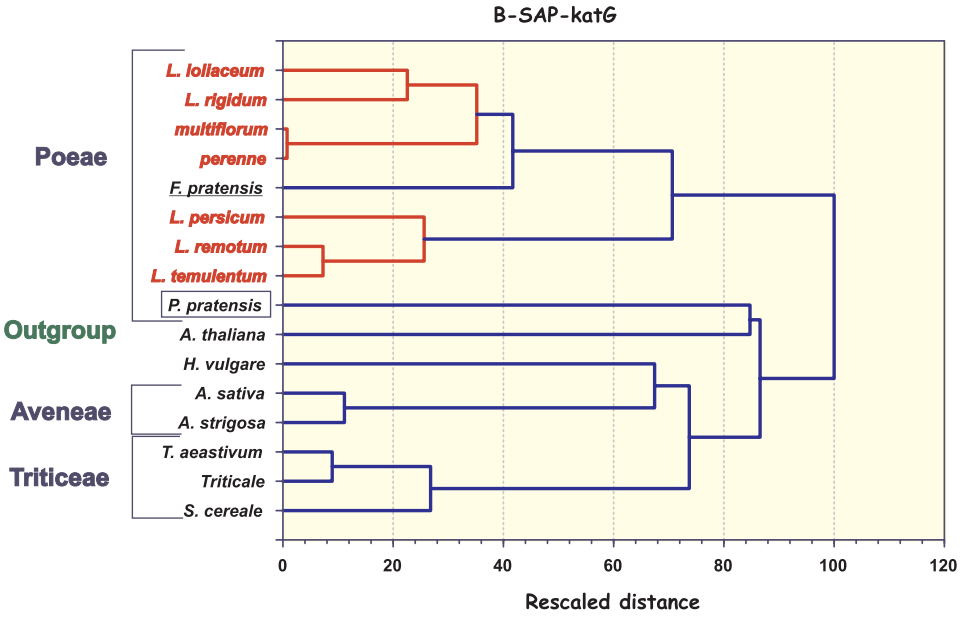


Figure 9.4. UPGMA grouping of „Core Pooids" based on B-SAP — katG markers
multiflorum — L. perenne ssp. *multiflorum, perenne — L. perenne* ssp. *perenne*

trivialis about 13 MYA. Timing of the divergence between Poeae and Triticeae is more difficult due to the lack of appropriate calculations however, taking into account the timing of the divergence between *Lolium* and *Hordeum* on 35 MYA (Huang at al. 2002) and between Erhartoideae (rice) and Pooideae (Gaut 2002) on 46 MYA, the divergence of Poeae from Triticeae can be estimated at circa 35-40 MYA. It is reasonable long time enabling *P. pratensis* to diversify both from *Lolium* and cereals. The above problem can be solved by sampling more species that are in between.

The overall genetic similarity between *Lolium* and cereals as well as between the Poeae tribe and cereals was low (I=0.584 and 0.612, respectively) reflecting their divergence. Molecular markers, considered individually and, in combination provide powerful characters for delineating analysed groups. It is important that the value of genetic similarities, to some extent, depended on the marker category (Table 9.1). It can have important implications when selecting markers for phylogenetic studies. The lowest values were revealed by all low copy sequences and multigene families (0.475 between *Lolium* and cereals, 0.500 between Poeae and cereals) thus, suggesting that these types of data can "produce" more species than exist. The difficulties in species separation using a single sequence are very often experienced. For example, it is impossible to resolve the genus *Lolium* using the *trnL-F* based tree (Catalan et al. 2004). Low genetic similarities revealed by low copy sequences should also be remembered when heterologous probes from cereals are used in genetic studies in *Lolium* or any other related taxa. Apart from the conserved gene order, the low copy sequences, not involved in the most important metabolic processes and thus not responsible directly for survival, can evolve relatively fast between closely related taxa. This phenomenon can be responsible for the difficulties in amplification and finding polymorphic STS loci suitable for genetic mapping studies in *Lolium*. Similarly, it can be a reason that anchor probes from *Medicago truncatula* do not work well in *Pisum sativum* (K. Polok, unpublished data). These findings do demonstrate that, apart from the overall similarity related with *Lolium* membership of Poaceae, *Lolium* and cereals genomes are rather different. Taken together several sources of information from phylogeny to comparative maps and sequences Gaut (2002) suggests that grass genomes are evolutionary labile, with less conservation than previously appreciated.

Table 9.1. The average genetic similarities between cereals, the genus *Lolium* and Poeae based on different marker categories

	Random, semi-random markers					B-SAP				STS			
	SSR	RAPD	ISJ	AFLP	SSAP	katG	IS 6110	rpo	pol	leu-tRNA	cereal STS	*Lolium* STS	*Lolium* pollen allergen genes
Lolium vs. cereals	0.34	0.66	0.49	0.72	0.71	0.61	0.69	0.71	0.76	0.51	0.41	0.35	0.63
Poeae vs. cereals	0.36	0.69	0.52	0.76	0.73	0.64	0.70	0.71	0.67	0.54	0.45	0.37	0.67

The B-SAP markers (katG, IS61110, rpo, pol), when taken together, are at the opposite extreme giving the highest values of genetic similarities (0.693 and 0.680, respectively). Indeed, it is not surprising because they have been selected as conservative markers with

low polymorphism within a species but enabling to reveal interspecific differences (Zielinski and Polok 2005; Krzakowa et al. 2007). At this point it is worthy to mention katG markers, for which genetic identities were lower (0.61 and 0.64) and comparable to those revealed by random markers. It is the other evidence confirming the high utility of katG markers based on catalase-peroxidase gene from *M. tuberculosis* in phylogenetic studies. Again these data are in agreement with mapping studies, in which katG markers have proved to be more effective than STS.

Random molecular markers, when several types are coupled together tend to give intermediate values of genetic similarities (0.584, 0.612) confirming that they represent a random sample of various genomic sequences. A cautionary point is that, if taken individually, some markers tend to underestimate genetic identities while the others overestimate. For instance, genetic similarities revealed by SSR were as low as 0.34 for *Lolium*-cereals comparisons and 0.36 for Poeae-cereals comparisons (Table 9.1). On the other hand AFLP and SSAP tended to give higher values (0.72 and 0.76 for AFLP; 0.71 and 0.73 for SSAP). The examples above demonstrate that all pro and cons should be considered when any single approach is selected for evolutionary studies. As it has been shown for the genus *Lolium* but also for cereals the best results are obtained when a lot of methods are taken together and consensus tree is accompanied by trees constructed from gene data. The other conclusion emerging from these studies is that using heterologous probes is not as straightforward as it appears from simple comparisons of gene order.

9.3.2. Similarity of genetic maps

Traditionally phylogenetic studies employ a single sequence and molecular markers to analyse a tremendous number of species representing different taxonomic levels to reconstruct the evolutionary history. However, the rapid generation of detailed genetic maps in many crops allows gene content and gene order comparisons when common DNA markers are employed. Comparative genetic maps of two species allow insight into the rearrangements of their genomes since the divergence of a common ancestor. The comparative mapping approaches show that the large majority of plant genes have close homologs within most other plants but also many rearrangements between related taxa can be observed. Synteny refers to the occurrence of two pairs of homologous genes on the same pair of chromosomes while colinearity indicates the conservation of gene order and content (Bennetzen 2000, Vision 2005). The Poaceae genome consists of conserved linkage blocks that evolved from a progenitor more than 70 million years ago and each grass genome can be reconstructed from 30 linkage blocks of the rice genome (Devos and Gale 2000). The genome of *L. perenne* consists from ten syntenic segments of Triticeae chromosomes, 12 syntenic segments of Aveneae chromosomes and 16 syntenic segments of Oryzeae chromosomes, suggesting a high degree of genome conservation. On the other hand ten large-scale chromosomal rearrangements are observed (Sim et al. 2005). The colinearity and synteny have practical importance as means for more effective dissection of agronomic traits. If a dense map exists for a given species, molecular markers or candidate genes can be used to manipulate a trait in another closely related species. But it is even more important for evolutionary studies. Some findings suggest that there may be a relationship between gene order

and phenotype (Vision 2005). Unfortunately, the majority of comparative studies employ low copy sequences whereas the rapid evolution is associated with intergenic sequences, often retrotransposons. Consequently, treating the chromosome as simply linear order of genes is a huge simplification.

Despite a number of success stories the comparative mapping is not as straightforward as previously thought. The analysis of comparative maps in Poaceae shows that the probability of two adjacent markers being syntenic can be as low as 50% (Gaut 2002). Typically, comparative maps have been constructed using heterologous RFLP probes from the closely related species (Gale and Devos 1998). The attempts to use this approach in *Lolium* have been undertaken by Jones et al. (2002b). It has been possible to establish colinear regions with Triticeae, but the problems have arisen in alignment of this map with the others, including the SSR map also constructed by these authors. The limitations of heterologous RFLP probes in different taxonomic families are that the 70-80% of sequence identity at the DNA level is demanded for such probes to cross-hybridize (Vision 2005).

The emerging approach likely to overcome the barrier of high DNA identity takes advantages from large expressed sequence tags (EST) or sequence specific tags (STS), which dozens are deposited in GenBank. The primers can be designed to conservative regions and then used to amplify a sequence in a species of interests. If it is possible to obtain a reproducible amplification pattern, preferentially a single band, and if it is possible to find polymorphism between parents of a cross then such ESTs or STSs can be mapped. Finally, putative homologs can be searched using appreciate software available elsewhere. Therefore, these mapped ESTs, STSs or any other sequences serve as anchor probes. The high level of sequence conservation within genes should offer the opportunity of comparing conserved genome organisation between species. Recently, more than 1 000 EST loci have been mapped in barley and they provide new anchor points for map comparisons (Stein et al. 2007). However, although the idea is simple, it is not easy to apply as it has been demonstrated in the mapping studies in *Lolium* (Chapter 6) and also experienced in *P. sativum* (K. Polok; unpublished data).

The STS sequences were selected from Taylor at al. (2001) based on the reported results showing restriction polymorphism in *L. perenne* ecotypes. Unfortunately, all STS proved to be useless in mapping studies due to no polymorphism between parents and the lack of segregation in F_2. Furthermore some problems were observed with the amplification of a specific band although it was possible to obtain reproducible multi-band profiles. The example includes ASN locus encoding L-asparaginase in barley. As reported by Taylor et al. (2001) comparisons between *H. vulgare* and *L. perenne* sequences reveal conservation in gene structure and overall similarity is 89%. The set of primers derived from asparagine synthetase (AS1) gene was able to amplify a single band comparable to that of *H. vulgare* but it was not polymorphic in spite of digestions with several different enzymes. Some difficulties were also encountered with RFLP probes from oat and barley. For example, cDNA clone BCD450 from barley was optimised to amplify two strong bands but they were twofold smaller than a band of Taylor et al. (2001). The bands were identical not only in parents of the mapping population but also in all studied species, thus suggesting the amplification of primers instead of a band. Eventually, lower annealing temperature resulted in multi-band pattern. In contrast to Taylor et al. (2001) results, reproducible amplification was received with oat probes CDO504 and

CDO1508. Unfortunately, no polymorphism was detected. The results of phylogenetic analyses suggest that the difficulties in using heterologous probes from cereals in genetic mapping in *Lolium* may arise from rather large evolutionary distance between these groups. Although it is thought that *Lolium* is closely related with cereals, the low genetic similarity between *Lolium* and cereals (0.594) in addition to about 35 million years of evolution are enough to cause problems in using STS from cereals. Noteworthy, the genetic similarity based on STS is even lower than estimated from the other marker categories. One can argue that the STS sample was too small however, the own experience from wide comparisons between *P. sativum* and *M. truncatula* makes plausible that genetic differences between some closely related taxa may be higher than it is thought. In a case of *P. sativum* about 50 STSs derived from *M. truncatula* were checked for polymorphism in *P. sativum* and the difficulties in obtaining specific amplification were even higher than in a case of *Lolium* and cereals. About 70% of primer sets gave only multi-band patterns, and the rest of them gave usually too short products that did not show restriction polymorphism between *P. sativum* cultivars and mutants (K. Polok, unpublished data). Thereby, the current results confirm the view that comparative mapping becomes more tenuous as the degree of relatedness decreases.

Notwithstanding the difficulties with STS markers the present map can be compared with the others using some enzymatic loci. The use of barley as a control in isozyme analyses enabled to connect *Lolium* enzymatic loci with those of *H. vulgare* (Table 9.2). Apparent co-location was observed for three enzyme loci, *Acoh*1, *Adh*1 and *Per*1 mapped on LG4 in *multiflorum* x *perenne* cross and reported on 4H in *H. vulgare*. Two enzymatic loci located on LG2, *Est2* and *Mdhp2* presumably corresponded to *Est2* and *Mdh2* locus on 3H in barley. In the latter species, *Est*2 locus is tightly linked with *Est1* and *Est4* loci (Liu et al. 1993). The similar linkage is not observed in *Lolium* and *Est4* is mapped on LG1. In a case of *Est1*, no polymorphism was found in all *Lolium* crosses and therefore it was not possible to map it. Unfortunately, wider comparisons were not possible due to the lack of barley molecular maps with many enzymatic loci. It is a pity, because the present data show that if suitable enzymatic loci are mapped they provide an easy way to align maps.

Transposon markers are located only on a few maps including barley and diploid oat. In total 78 retrotransposons (*BARE-1*; *BAGY-2* and *Suk*) are mapped on the Streptoe x Morex barley genetic map and 65 *BARE-1* transposons on the *A. strigosa* x *A. wiestii* genetic map (GrainGenes 2007). In comparison with the current *multiflorum* x *perenne* map, this number is 20-30% smaller. The percentage of transposon markers in comparison with all mapped markers is somehow higher for *H. vulgare* than for the other two species (Figure 9.5). It is likely to be a function of total mapped markers. The smallest number of markers is on the *H. vulgare* map (313) in comparison with *Avena* and *Lolium* (502 on both maps). If the distribution of transposons is compared, the clear pattern is that they are rather equally distributed over the majority of linkage groups, although in each species at least one group has much fewer transposons than the others (Figure 9.6). In *Avena* no transposon sequence is observed on 2A; only 4% of transposons can be observed on LG7 of *Lolium* while 6% can be found on 4H of *Hordeum*. The transposons are equally distributed on the remaining linkage groups in *Lolium* and *Hordeum*. In contrast, differences are observed in *Avena*, the transposons tend to cluster on 5A (29%) and 7A (23%). The different pattern of the transposon distribution may reflect the evolutionary relationships between all species but it is also likely

Table 9.2. Location of enzymatic loci on *multiflorum* x *perenne* and *H. vulgare* genetic map

Enzymatic loci	*multiflorum* x *perenne*	*H. vulgare*
Acoh1	LG4	4H
Adh1	LG4	4H
Aat2	LG1	6H
Cap2	LG2	7H
Est2	LG2	3H
Est3	LG1	1H
Est4	LG1	3H
Gtdh2	LG5	7H (?)
Mdh2	LG4	3H
Mdhp2	LG2	-
Per1	LG4	4H
Per3	LG6	2H
Pgdh1	LG1	5H

Based on Brown 1983; Franckowiak 2007; Kleinhofs et al. (1993); Liu et al. (1993)

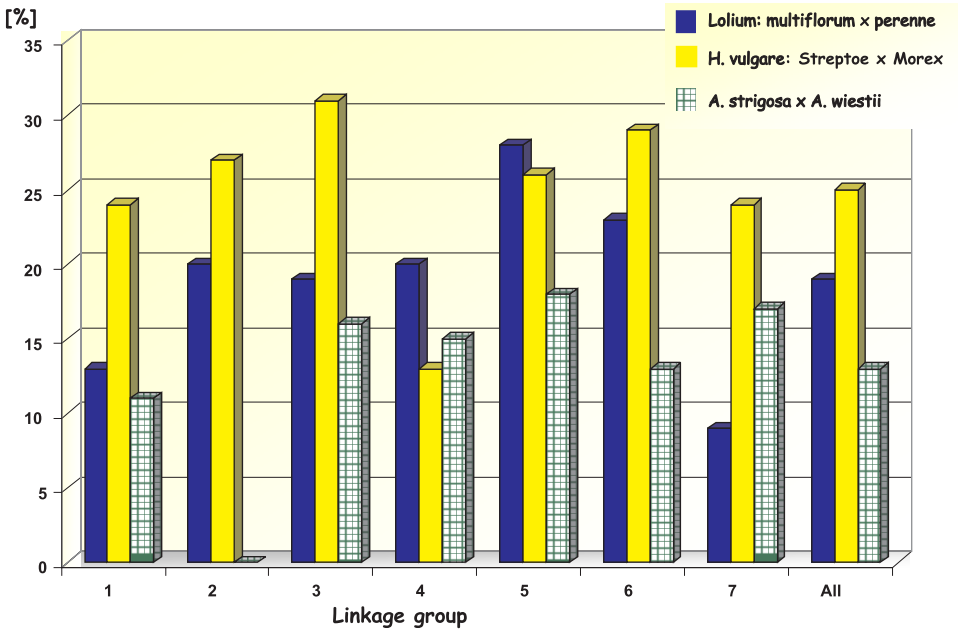


Figure 9.5. Transposon abundance on genetic maps of *Lolium*, *Hordeum* and *Avena* in comparison with all mapped markers
Hordeum and *Avena* maps were taken from GrainGenes: A database for Triticeae and *Avena* (2007)

that it results from the different transposon sampling strategy. The confirmation of the above relationships is pending more transposons mapped in different cereals.

To summarize, notwithstanding the difficulties in anchor probes, and whether comparative mapping are useful or not for breeders, comparisons of gene order and gene rearrange-

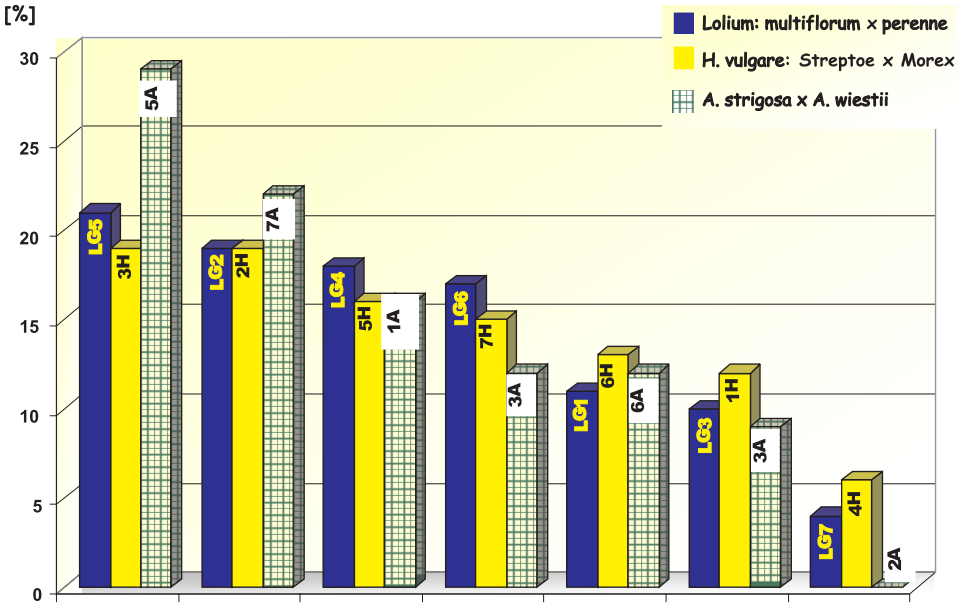


Figure 9.6. Distribution of transposons among linkage groups of *Lolium*, *Hordeum* and *Avena*
Hordeum and *Avena* maps were taken from GrainGenes: A database for Triticeae and *Avena* (2007)

ments are invaluable for studying the evolutionary mechanisms at the most basic level. The dense genetic map like this constructed within the present work is just the first step to undertake the challenge of studying the evolution of the gene order and gene content. But it is a crucial step without which it would be impossible to learn more about exiting processes by which genomes evolve.

9.4. CONCLUSIONS

1. The low genetic similarity between the genus *Lolium* and cereals demonstrates the distinctiveness of these two groups.
2. The most reliable phylogenetic tree can only be constructed with help of many marker categories, however gene trees can be useful in tracing introgression.
3. The tree based on katG markers proves to be highly useful in resolving phylogenetic relationships between *Lolium* and cereals. They can be used as an alternative to multi-marker approaches.

4. The difficulties in using anchor probes from cereals in *Lolium* genome mapping result from low genetic similarity between both groups and demonstrate that, apart from the overall similarity related with *Lolium* membership of Poaceae, *Lolium* and cereals genomes are rather different.

10. MARKER EFFICIENCY

10.1. INTRODUCTION

It is not overstatement to say that any genetic analysis would be impossible without genetic markers, whether morphological or molecular. They lend themselves to a wide variety of methodological approaches depending on the particular problem and research goals. Their generic applications include population, and evolutionary genetics as well as many aspects of applied genetics including genome mapping, analysis of quantitative traits, gene isolation and many others. Isozymes introduced in the mid 1960s, were the first markers that provided insights into microevolutionary processes but also they became useful as a marker system in the analysis of experimental crosses. Invention of the polymerase chain reaction (PCR) revolutionized molecular biology and has stimulated many new methods of genetic marker acquisition. These new methods that have been emerging in between eventually gain wide popularity. But, usually, after a period of enthusiasm, a wave of interest has focused on others newly introduced methods. Typically, the earlier methods have not been abandoned however, their usage sometimes have been limited. The example involves protein electrophoresis that remains a simple and useful method, although opinions that it is old fashioned and should be abandoned do exist. Nevertheless, the easy interpretation of electrophoretic patterns (zymograms) and codominance favour it in comparison with many DNA markers. Similarly, a lot of objections are elevated for different PCR-based techniques. Consider RAPD markers that are thought to be irreproducible and not highly polymorphic despite many valuable results obtained through their usage. Examples include the first genetic map of *Lolium* (Hayward et al. 1998), and molecular phylogeny of the grass genus *Brachypodium* (Catalan et al. 1995). In the latter studies RAPD markers proved to be more efficient than RFLPs.

In deciding which marker system is the best for a given application several key factors should be considered. The level of polymorphism, the availability of plant material necessary for the analysis and the costs are among the most important. There are many manuals and guides how and when to use a given type of molecular markers. Although they offer quite useful reviews for beginners they do not avoid popular opinions, not always grounded very well and repeated over and over again. The issue is exactly in what is meant by own experience. In the present work a huge amount of different laboratory techniques has been addressed at different levels of analyses from genetic diversity studies, phylogenetic analyses and mapping. These data enabled to estimate the usefulness, efficiency and limitation of different types of markers in variety genetic analyses. This chapter summarizes the results obtained for *Lolium* with regard to marker efficiency and costs. It also proposes the most

obvious applications taken into account the different technological input necessary for the development of each marker category. The usefulness of each marker category is presented in the three important areas of genetic research - these areas which were also touched in the studies on *Lolium* evolution.

10.2. COSTS OF MARKER ASSAYS

When starting any analysis, everyone would like to obtain as much information as possible at as low costs and personal efforts as possible. Hence, it is not surprising that the first thing to consider is the number of markers (bands) that should be generated and techniques that are the cheapest with respect to input and information retrieved. Despite chemicals needed for a particular reaction, matrices through which small DNA or protein fragments migrate should be considered as well. The usual electrophoretic media are agarose and polyacrylamide gels for both DNA and proteins in addition to starch for the latter. From a technical point of view, any marker system that requires polyacrylamide gel electrophoresis is more challenging that those based on agarose or starch gel. On the other hand, polyacrylamide gives the highest resolution, but not always it is necessary. Notwithstanding these methodological considerations, another popular opinion is that enzymes are the cheapest while DNA markers such as AFLP or transposon-based (SSAP) belong to the most expensive. It is true if total costs per a sample are taken into account (Figure 10.1). Roughly, a single AFLP or SSAP reaction together with costs of a gel and staining is almost sevenfold more expensive than enzyme or DNA analyses such as RAPD. However, the proportion becomes inverted if the total number of revealed loci is considered. To obtain 100 bands or loci one should pay about 10 euros if AFLP or SSAP markers are used but 100 enzymatic loci would cost about 40 euros if it was possible to obtain (Figure 10.2). Indeed, even RAPD markers are more expensive then AFLP/SSAP. The analysis of cpDNA and mtDNA, so popular in phylogenetic studies, also is not cost effective especially when coupled with restriction digestion. These differences are even more dramatic if polymorphic loci are taken into account (Figure 10.3). It is obvious from these comparisons that such techniques as AFLP or SSAP are the most cost effective. Another advantage is that the huge number of markers can be obtained with less man power. A single AFLP or SSAP gel contains from 50 to 100 bands, so only a few analyses are needed to sample a large portion of loci. For example, a single RAPD primer can generate up to seven loci, among which about six can be polymorphic (Figure 10.4). In comparison, a pair of SSAP primers can give from 60 to 90 bands and about 40 to 80 are polymorphic (Figure 10.5). So it is feasible to assay hundreds of individuals for a hundred of polymorphic loci in a week or so without much effort. On the other hand, they need a polyacrylamide gel and the start-up costs are quite high. In a case the AFLP or SSAP are too demanding and agarose gel should be used instead, the markers based on junctions between introns and exons (ISJ) are recommended due to relatively high polymorphism. Nonetheless, RAPDs are more cost effective that cpDNA or enzymes. To this point the possibility to obtain enough DNA may be a certain limitation (Figure 10.6). Although amount of DNA per a reaction is exactly the same for RAPD and ISJ, the latter markers are more polymorphic and hence, total amount of DNA to obtain 100 polymorphic loci is lower.

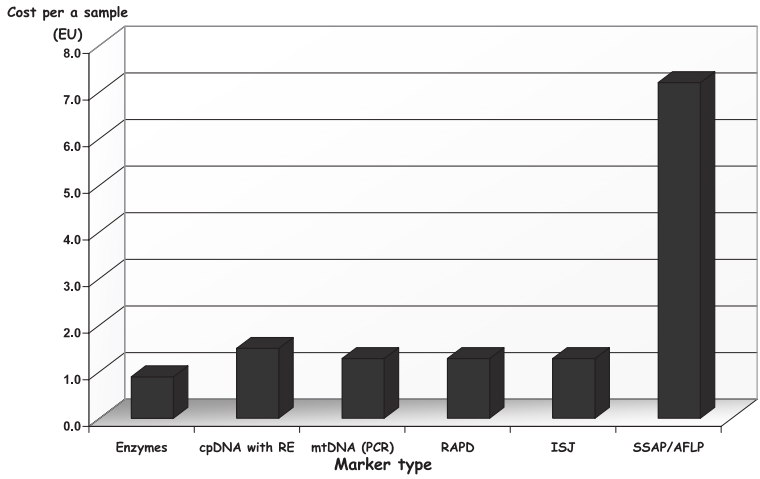


Figure 10.1. Total cost of a given marker type per a sample

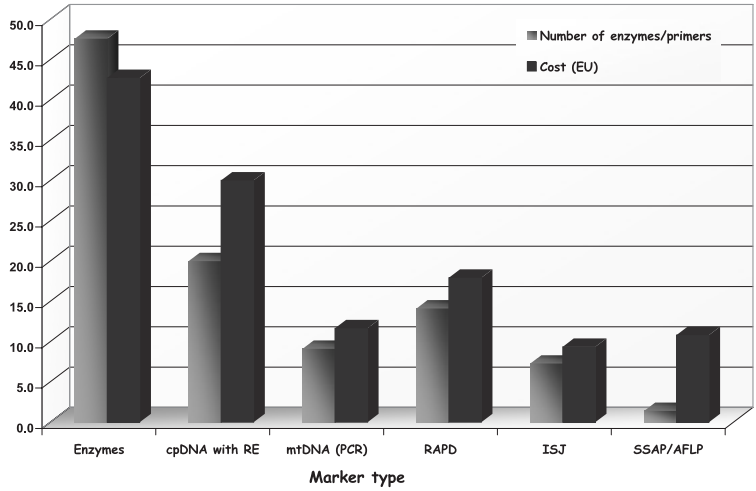


Figure 10.2. Number of primers needed and costs to obtain 100 bands/loci per a sample

10.3. DNA PURITY AND MARKER REPRODUCIBILTY

The high purity of DNA is sine qua non to obtain reproducible results for any marker category. The common view is that some methods perceived as less sophisticated (RAPD or similar) do not need high purity of DNA and consequently many rapid protocols and commercial kits are used. On the other hand RAPDs have developed a reputation of poor reproducibility. As a result, having bad own experience, many reviewers reject papers based on RAPDs as unreliable. Conversely, own experience was that 100% of reproducibility can be obtained provided DNA is of high quality. Every time the isolation procedure was simplified, the results were much worse. Generally, commercial kits are not suitable for isolation. Instead

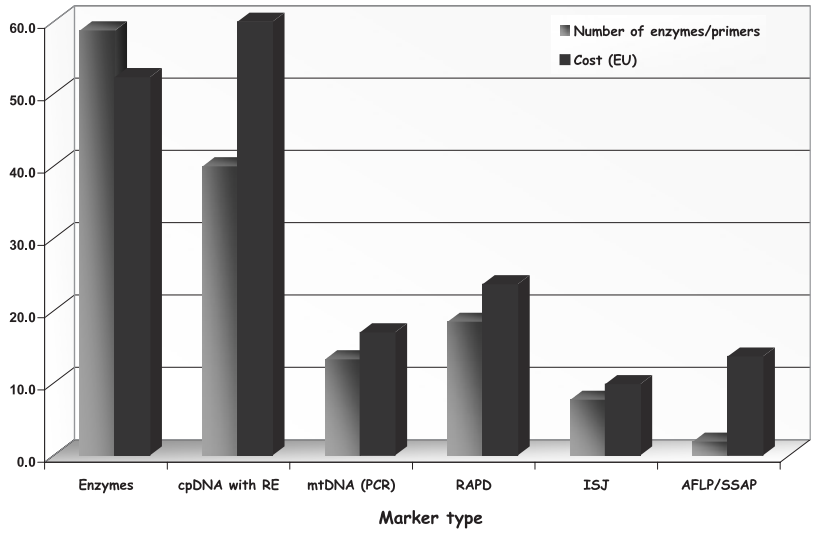


Figure 10.3. Number of primers needed and costs to obtain 100 polymorphic bands/loci per a sample

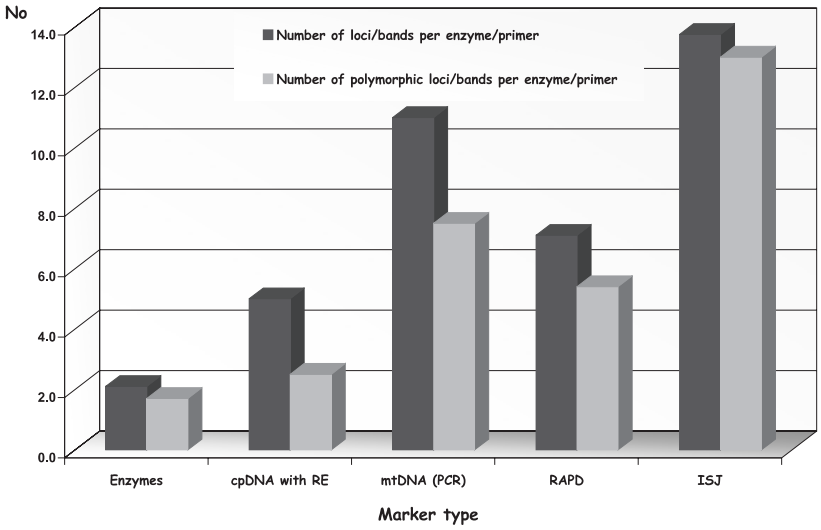


Figure 10.4. Efficiency of molecular markers

the CTAB method with additional cleaning by cesium chloride should be used. For more troublesome species including liverworts, pines, roses the addition of PVP is recommended to reduce the level of phenols that have tremendous effect on RAPD quality. Note, that getting the procedure longer than in the original protocol e.g., precipitation time lengthening, precipitation with ethanol instead of isopropanol improves considerably the DNA purity and quality. Once DNA is isolated it can be used for many purposes, and the RAPD can be the simplest

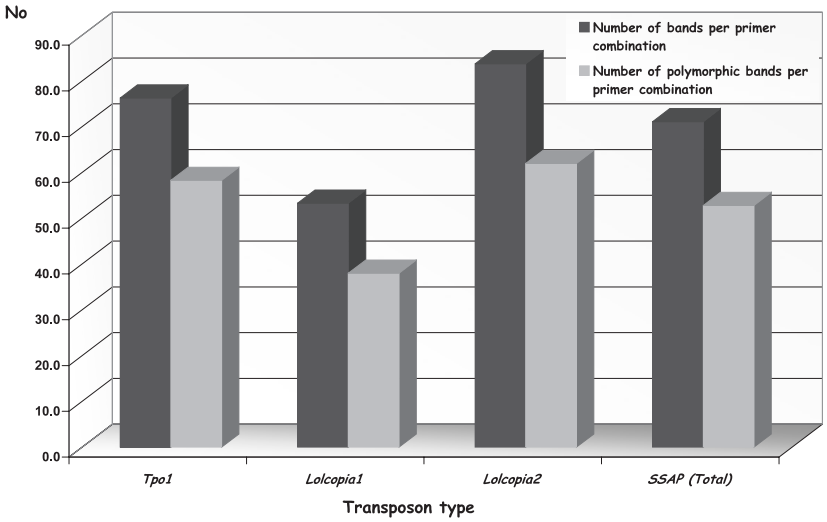


Figure 10.5. Efficiency of SSAP markers

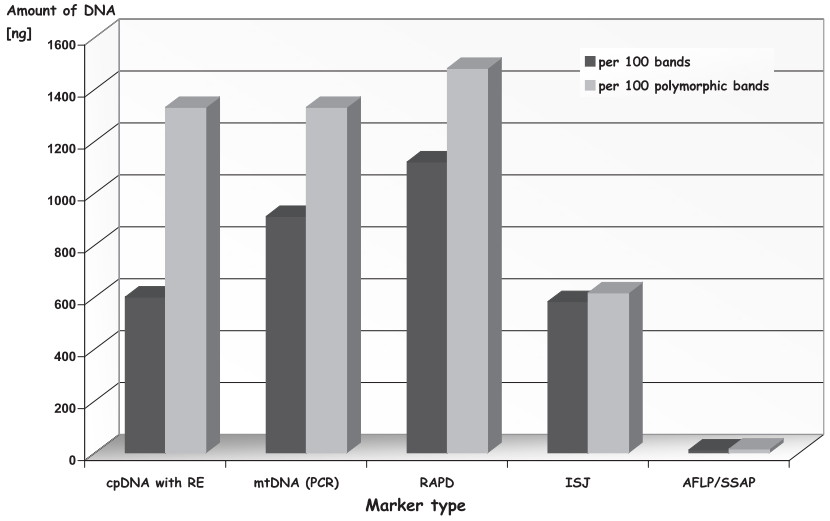


Figure 10.6. Amount of DNA needed for analysis of 100 bands and 100 polymorphic bands per a sample

method. However, the requirement of pure DNA makes the RAPD more demanding and longer technology than previously intended. Another misunderstanding involves the amount of DNA. Almost each manual states that only few nanograms (10-25 ng) of DNA are needed for RAPDs in comparison with about 100 ng in a case of microsatellites (Application...1998; Mutant...2002). Nothing more wrong. In principal, PCR reactions are inhibited strongly if DNA is contaminated by polysaccharides. Because rapid isolation protocols are used for RAPDs, DNA is usually contaminated and its amount should be lowered to obtain any amplification.

But the immediate result is low reproducibility. With pure DNA about 80-100 ng is sufficient amount for high reproducibility. Less amount of DNA decreases the reproducibility of RAPDs but not only. For example, it is impossible to obtain reproducible IRAP (Inter-Retrotransposon Amplified Polymorphism) fingerprints in *P. sylvestris* with as little as 20 ng of DNA. Increasing the amount of DNA up to 80 ng improves greatly the performance of this kind of markers (K. Polok, unpublished data). In summary, for RAPDs as for any other marker type, the true is that they can find applications in all areas of genetic research as long as they can be suitable analysed.

A by-product of the present analyses is comparison of silver staining and autoradiography for resolving polyacrylamide gels. The most obvious is that silver staining is somehow tricky and should be properly done to receive good results. Again, in most cases commercial kits are not the best solution. In contrast, autoradiography is less prone to mistakes but has some problems, with hazardous reagents, and as compared with silver staining it proves to be less reproducible. The “ghost bands” or faint bands difficult to interpret are very often observed. The other advantage of silver staining is easier extraction of bands from the polyacrylamide gel. The re-hydratation of a given band for as short as 10-20 minutes is enough to obtain 5-10 μg of high purity DNA suitable for re-amplification, cloning and others manipulations.

10.4. WHICH MARKER TYPE FOR POPULATION GENETIC ANALYSES?

Population genetics is a field in which many individuals are analysed. Thus, the marker system should be fast and relatively cheap. Even many advanced methods have been developed for population genetics studies, isozymes still offer an alternative option owing to simplicity and easy viability. The another important feature is that for many years of studies statistical methods have been elaborated for isozyme analyses and they have proved to resolve correctly genetic structure and phylogenetic relationships in many organisms at different taxonomic levels. The whole theory and methodology of genetic identity assessment has been developed for enzymes. For any DNA markers such enormous amount of data about population structures and genetic diversities from a wide range of organisms has not been gathered yet. Therefore, many traditionally trained population geneticists treat DNA-based parameters with reserve raising the problem of homoplasy that muddy the historical record. To ascertain that protein and DNA-based methods describe the same pie of population history, a wide analyses employing different assays and using the same material are necessary. Until sufficient amount of comparative data is available some caveats to replace traditional methods with DNA analyses will exist. The data presented in the Chapter 4 belong to the broadest studies comparing enzymes with DNA markers with respect to population genetic parameters using the same material and thus, can guide the methodological choices in similar studies. One emerging consensus seems to be that the most of marker categories give similar results and that the parameters of genetic diversity are comparable to those obtained by means of isozymes (Table 10.1). However, enzymes demonstrate somehow higher polymorphism as indicated by the number of polymorphic loci and gene diversity. A little better efficiency of enzymatic markers is exemplified by their first rank with respect to number of

alleles per a locus and gene diversities (Table 10.2). This argues for their further applications every time when DNA analyses are troublesome, for exotic species, rich in compounds making difficult DNA isolation or simple at the first stages of studies, at which the overall view is important. For example, on the area of Poland, the moss, *Pleurozium schreberi* (Wachowiak-Zielinska and Zielinski 1995; Zielinski and Wachowiak-Zielinska 1995) reproduces only vegetatively. The isozyme analysis was made first to have an idea about its clonal structure. These early results revealed the enormous genetic diversity at the enzymatic level, so the studies were broaden to thousands of individuals and with such a big number they can only be accomplished using protein electrophoresis. Another nice example involves *Pellia species* that are difficult to separate based on morphology. The simple starch gel electrophoresis and only one cheap enzymatic procedure, peroxidase staining enables unequivocally to identify all taxa. From the other markers, it is worthy to note ISJs and RAPDs that produce results nearly the same as enzymes. As for RAPDs, in spite of many objections found elsewhere in the literature, if properly done, they do offer a quick way of screening potential molecular markers from many loci. However, taking into account that DNA of high purity is needed and the isolation can take from a day to few, none marker system can be compared with the enzymes in respect to their simplicity and availability, with seconds to obtain extracts. Finally, organelle markers are more useful for studying phylogeographic structure than for genetic diversity itself. On the other hand transposons are invaluable if one is interested in the tempo of microevolutionary processes and adaptations to changing environmental conditions. The movement of transposons is between the first signs of speciation.

Table 10.1. Comparison of genetic variation parameters in *L. perenne* (ssp. *multiflorum*, ssp. *perenne*) based on different molecular markers

Method	Parameter								
	P	*A*	N_e	H_T	H_S	D_{ST}	G_{ST}	F_{IT}	F_{IT}
Enzymes	**80%**	1.86^{a}	1.57^{a}	0.342^{a}	0.271^{a}	0.117	0.340^{b}	0.207^{c}	0.002^{b}
cpDNA	45%	1.45^{d}	1.32^{de}	0.172^{f}	0.070^{c}	0.118	0.249^{b}	0.394^{bc}	0.383^{a}
mtDNA	*52%*	1.53^{cd}	1.38^{bcd}	0.214^{de}	0.078^{c}	0.131	0.303^{b}	0.502^{ab}	$0.384a^{b}$
RAPD	64%	1.73^{ab}	1.44^{bc}	0.254^{bc}	0.111^{bc}	0.160	0.590^{a}	0.565^{ab}	0.004^{b}
ISJ	**82%**	1.82^{a}	1.46^{b}	0.270^{b}	0.271^{b}	0.161	0.530^{a}	0.496^{ab}	0.022^{b}
Tpo I	61%	1.61^{bc}	1.37^{cd}	0.235^{bcd}	0.078^{c}	0.157	0.369^{b}	0.669^{a}	0.007^{b}
Lolcopia I	*51%*	1.51^{cd}	1.39^{bcd}	0.184^{ef}	0.064^{c}	0.120	0.289^{b}	0.657^{a}	0.005^{b}
Lolcopia2	58%	1.58^{cd}	1.46^{b}	0.225^{cd}	0.079^{c}	0.145	0.341^{b}	0.645^{a}	0.002^{b}
SSAP - total	57%	1.56^{cd}	1.29^{e}	0.214^{de}	0.074^{c}	0.141	0.333^{b}	0.657^{a}	0.005^{b}

[a]different letters mean significant differences at P=0.05

10.5. MOLECULAR MARKERS FOR GENOME MAPPING

The mapping studies are different from all the others in a sense that each gene with known function is worthy to map. This is to say that it is necessary to make efforts to map even a single enzyme. After all, each DNA marker is only a sequence with unknown function and presumably, it represents a repetitive DNA. Notwithstanding the above prerequisite, whether to use or not any marker category depends on goals of genome mapping. Whenever

Table 10.2. Rank of marker systems based on genetic parameters values

Method	Parameter								
	P	A	N_e	H_T	H_S	D_{ST}	G_{ST}	F_{IT}	F_{IT}
Enzymes	2	1	1	1	1	9	5	9	9
cpDNA	9	9	8	9	8	8	9	8	1
mtDNA	7	7	5	7	6	6	7	6	2
RAPD	3	3	3	3	3	2	1	5	7
ISJ	1	2	2	2	2	1	2	7	3
Tpo1	4	4	6	4	5	3	3	1	4
Lolcopia1	8	8	9	8	9	1	8	2	5
Lolcopia2	5	5	4	5	4	4	4	4	8
SSAP - total	6	6	7	6	7	5	6	3	6

Shadow boxes indicate the group of markers with the highest values of genetic parameters at P=0.05

a map is used for QTL mapping high resolution is necessary. The other strategy is employed if a single gene should be mapped. Then BSA (Bulk Segregant Analysis) can be used to find as many markers as possible in a given region. Once markers are found a detailed map for that region is constructed. For species comparisons there is always a choice between high resolution of a single map and mapping in many cross combinations. It is possible to use only selected sequences or isoezymes in a case of preliminary species comparisons. The use of common sets of DNA probes to detect and map homologous sequences across different species has revealed a high degree of conservation in species belonging to the same family. The extended comparative relationships facilitate the transfer of genetic knowledge from well-studied crops to other species. Unfortunately, the use of common DNA probes or STS markers is not free of problems. Own results have demonstrated that STS markers derived from cereals are completely useless in genome mapping of *Lolium* owing to low polymorphism or inability to amplify a single PCR product. Similar results were also obtained for STS markers derived from *Medicago truncatula* and applied for genetic mapping of stiff straw in *Pisum sativum* (K. Polok, unpublished results). All primers are not able either to amplify a discrete band or they do not reveal polymorphism between parents, cultivars and mutants. Surprisingly, it is easier to obtain reproducible results using primers derived from bacterial genes than those from close relatives. Because bacteria-derived markers tend to be linked with enzymes it seems reasonable to incorporate them in maps as potential markers of genes as well as potential markers for interspecific comparisons. Comparisons of the utility of different marker categories as indicated by the number of primers needed for a map construction in *Lolium* and the number of polymorphic loci between parents demonstrate the relatively high utility of RAPDs and ISJs. Unexpectedly, quite poor performance is observed for AFLP that are thought to be the most reliable and informative markers for genetic mapping (Table 10.3). Unfortunately, own results do not confirm this view, also because they tend to group and they are underrepresented as markers linked or flanking QTLs. This feature may seriously limit their utility for mapping quantitative traits. Perhaps it is the reason for difficulties in alignment of most *Lolium* maps based on AFLPs.

The SSR markers are highly polymorphic and they are useful in mapping studies. The disadvantage is the effort needed for their development in each species and relatively high

Table 10.3. Characteristics of primers used in map construction of *Lolium* (*multiflorum* x *perenne*)

Parameter		Method							
		RAPD	ISJ	SSR	AFLP	SSAP	STS	B-SAP	Total
Tested primers (or pairs of primers)	N°	60	12	7	36	6	13	15	147
Primers used in map construction	N°	12	7	5	6	4	0	5	43
	%	20.0	58.3	71.0	16.7	66.7	0.0	33.3	29.3
Total n° of identified markers	N°	164	97	10	377	192	33	45	1018
Polymorphic markers between parents	N°	86	65	6	49	34	0	24	264
	%	52.4	67.0	60.0	12.9	17.7	0.0	16.6	25.9

proportion of distorted markers. Another problem is related with the effort necessary to map a suitable high number of SSR markers. Typically each PCR reaction gives rise to mapping only a single marker. To map, let say 16 markers, 16 PCR reactions should be made. One PCR plate carries 96 reactions. Thus, a population of 100 individuals can be analysed within 16 days provided a reaction per a day. Even enzymatic markers are more time effective. One starch gel carrying about 25 individuals can be sectioned into five replicate slices, and each slice can be incubated with a different stain. Two gels per a day can be run without much effort. Thus, a total of 100 individuals could be scored for 16 enzymes, each giving a single polymorphic locus, within just 8 days.

The high level of distortions can limit the utility of transposon-based sequences, but on the other hand they are highly valuable for mapping species boundaries. Again, quite surprising results were obtained in a case of RAPDs. Among all marker categories they exhibit quite high polymorphism, low level of distortions and quite uniform distribution over the whole genome. The emerging view is that RAPD markers represent the most random sample of different genomic sequences. That is why they are very useful in filling the gaps in maps created by AFLP clusters. Comparing the effort needed to develop AFLPs and RAPDs, the latter are also advantageous. In total six AFLP primers generated 137 bands that were mapped. In a case of RAPDs these numbers are 12 primers and 132 bands. Although twice as many RAPD primers are needed the simplicity and rapidity of the analysis make them advantageous over AFLPs. However, the most obvious conclusion from the mapping studies is that several different marker categories are prerequisite for high resolution and uniform distribution of markers. In any event, such multi-marker maps are necessary for evolutionary considerations. It would be impossible to conclude about species boundaries between ryegrasses if only AFLPs or RAPDs were used. This is again to agree with old views of traditional geneticists that each approach based on a single methodology tends to produce biases.

10.6. PHYLOGENETIC AND EVOLUTIONARY STUDIES

Phylogenetic studies are predominated by sequencing of specific genes. In fact these analyses are mainly by-products of the other studies and the main obstacle is that all information is based usually on a single individual as a species representative. This is because is much more difficult to analyse many individuals from many species especially based on sequencing data. Molecular markers offer important improvements and they represent a rather

random selection of different sequences. Similarly, to population genetic and mapping studies, the surprisingly good results were obtained with random markers including AFLP, ISJ and RAPD. Conversely, the organelle DNA and STS markers derived from cereals tend to produce biases. The present data confirm that the best resolution is obtained if many different sequences are used together. The consensus tree based on almost 3000 molecular markers mirrors very well evolutionary relationships within Poaceae. However, it seems unrealistic to employ so many markers in routine evolutionary analyses in which tens and hundreds species are sampled. To this point, markers based on bacterial genes seem to offer a good alternative. Their trees are very alike those based on multiple markers but their development needs less efforts.

B-SAP - a new approach for phylogenetic studies

The idea is based on using primers complementary to bacterial genes to amplify plant DNA and reveal polymorphism. For the first time B-SAP was tested using several representatives of Poaceae (Polok 2005; Zielinski and Polok 2005). Several primer categories can be used, however the best results, transferable among many taxa are received through using primers complementary to the *M. tuberculosis KatG* gene encoding catalase-peroxidase and primers complementary to IS6110 element from the same species. The revealed markers are dominant i.e., the presence of a band is dominant over its lack. They are characterized by low polymorphism within species, however great differences are observed at the interspecific level. The mapping studies demonstrated that katG markers are linked with peroxidase and some other enzymes, thus confirming that they are related with coding sequences, presumably of bacterial origin. By contrary, markers derived from IS6110 behave alike transposons. What is more, all bacteria-derived markers are rarely distorted in comparison with the others. The percentage of bands that deviated from the expected Mendelian ratios is below the mean for the whole genome. Therefore, it is reasonable to incorporate them in mapping studies as well as in phylogenetic applications. We propose to use them in phylogenetic studies to resolve deeper evolutionary relationships as well as species specific markers. Because they are based on bacterial sequences, we propose to ascertain them as **Bacteria Specific Amplification Polymorphism (B-SAP)** with a gene name if necessary.

10.7. CONCLUSIONS

1. If properly done each marker category produces reliable results and can be used in any genetic analysis.
2. Enzyme electrophoresis remains the simplest and fastest method for population studies. However, more advanced marker systems are most cost effective if number of polymorphic loci is taken into account.
3. The best resolution of genetic maps is obtained when several different marker categories are used. The different markers are especially important if a map is used for evolutionary considerations.
4. Phylogenetic analyses based on many categories of markers mirror evolutionary relationships the best however, such analyses can be unrealistic for many species at once.
5. B-SAP markers offer a good alternative for multi-marker approaches in evolutionary considerations due to reliability and simplicity.

11. CONCLUSIONS ABOUT EVOLUTION OF THE GENUS *LOLIUM*

The molecular perspectives offer new insights into old problems - what processes are responsible for enormous genetic diversity, how do species evolve and what is their future. It is now undeniable that evolution is a genetic process and all changes have their sources in the marvellous genome plasticity. This up-to-now hidden the “genome world” has been opening alongside with the progress of molecular technologies. The present studies on the genus *Lolium* emphasized how these modern methods can enrich our understanding of evolutionary processes. However, they can not replace all traditional approaches to the study of species. This is the phenotype undergoing selection and each new combination of genes has to be checked in changing environments. Think back to just an example of transposons whose movement can drive speciation by inducing polymorphism and creating new genes, but only if their movement is not harmful for genes and for the whole organism. There is a kind of homeostasis between “novelty” created by transposons and well tried combinations. Just like in the evolutionary studies. There would be no possibility to study *Lolium* and any other taxa without previous knowledge about their distribution, morphology, ecology and even history. Molecular methods are useless without prior identification and classification of phenotypes. Therefore all molecular techniques can be the best used when coupled with the more traditional methods, sometimes perceived as old fashioned. The genus *Lolium* is one of many examples, in which the evolutionary history could be elucidated due to combining different appraisals and using huge knowledge gathered previously by botanists, breeders and all these anonymous people who spent their life for studying species. Drawing extensively on this heritage and making the best use of all new molecular approaches from the field of population and evolutionary genetics as well as genome mapping, the phylogenetic relationships within the genus *Lolium* can be presented in a new light.

Overall, the genetic analyses documented very well the lack of reproductive barriers between *L. multiflorum* and *L. perenne*. Studies employing genetic mapping and QTL analyses have shown that differences between these two species are nothing more than intraspecific variation at least partially similar to that observed between crop cultivars. When coupled with historical data it has became obvious that this variation is related to domestication processes, whose nature is like that diversifying teosinte and maize several thousand years ago. Huge variation at 145 QTLs associated with 21 traits, and increased mobility of transposons are tangible evidences supporting this view. The most important conclusion from this part of studies is that the classification proposed by the Integration Taxonomic System of the USA should be commonly accepted and *L. multiflorum* and *L. perenne* should be classified as subspecies i.e., *L. perenne* ssp. *multiflorum* and *L. perenne* ssp. *perenne*.

Apart from *L. multiflorum* and *L. perenne* the present molecular studies based on enormous number of 2984 molecular markers representing various methodologies have prompted major revisions of thoughts concerning evolutionary relationships within the genus *Lolium*. It is now clear that the progenitor of the genus was common to *Lolium* and *F. pratensis*, had the closest affinity to *Schedonorus* taxa and gave rise to two lineages, of which diversification began 2.7 MYA. Today they are represented by the Temulentum clade consisted from autogamous annual species, *L. persicum*, *L. remotum* and the best known member of this clade - L. temulentum. *L. persicum* was the first species that differentiated about 1.3 MYA, then the remaining two species were split about 0.95 MYA. Nevertheless, as weeds of crops, all of them might have been domesticated unconsciously in days of the primitive agriculture, circa about 10 000 years ago.

The second lineage is represented by the Perenne clade that involves allogamous species *L. perenne* with two subspecies, annual or biennial *L. perenne* ssp. *multiflorum*, perennial *L. perenne* ssp. *perenne*, annual *L. rigidum* as well as autogamous *L. loliaceum*. This clade diverged from the common ancestor with *F. pratensis* about 2.35 MYA. The earliest diverged species was *L. rigidum* that split from the others about 1.4 MYA. *L. loliaceum* evolved through several point mutations related with the transition from allogamy to autogamy and its divergence from *L. perenne* can be dated to 1.2 MYA. The populations of *L. perenne* started to diversify during the Tertiary and Quaternary glaciations. It is likely that the perennial forms came into existence 167 000 years ago as a response to more harsh environment. Further diversification has been prompted by the domestication process that started before twelfth century in Italy, of which we are eyewitnesses today. A spectacular discover is that transposons are the most important driving forces of evolutionary processes observed in *multiflorum* and perenne. It is elegantly shown by their higher mobility in *L. perenne* ssp. *multiflorum* observed in genetic diversity studies, strong distortions from the expected Mendelian segregation ratios and their association with the QTLs noticed in mapping studies.

From these wide molecular surveys one the most important notion emerges that the evolutionary properties of any given group of organisms could be characterized by a number of methods addressing to different levels of the same phenomena. Only then the reasonably clear picture of genetic diversity can be obtained taking into account the present development of technologies. Among many technologies used in the studies on *Lolium*, it is worth mentioning markers derived from bacterial sequences (B-SAP). It was a great surprise to be able to amplify plant DNA using primers specific to so distant organism as *M. tuberculosis*. The astonishment was even greater when it was possible to map markers derived from catalase-peroxidase of *M. tuberculosis* in close proximity to peroxidase loci and the other enzymatic loci. This example demonstrates how fascinating and strange can be the evolutionary roads. And for the first time, we have tools which, if coupled with advance statistics and traditional morphology have potential to reveal secrets of evolutionary changes and help us to understand and protect enormous diversity of living creatures. And last but not least, new methods that will emerge in the nearest future will certainly add a new look at the genus *Lolium*, at any other taxa and at the evolution of life on the Earth.

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Journal abbreviations according to ISI

13. SUPPLEMENTARY MATERIALS

In the studies variety of approaches and materials were used to address questions about genetic diversity and evolution of the genus *Lolium* as well as to elucidate the genetics of chosen characters. Many of them were modified or even new techniques were introduced. Researchers employ molecular techniques, modify them and it is a good practice to present a detailed methodology in each paper. It is invaluable for anybody who would like to follow the procedures for own studies or to follow the presented work in more details. On the other hand, such a description of methodology in a book may be perceived as somehow schoolish and unnecessary or even making difficult to follow the results, which are lost in the sea of details. In the high quality journals quite a new trend has been observed more and more frequently. This is a limitation of technical descriptions to those absolutely necessary to understand the text and logic of an experiment while the details are provided on line for everyone interested in them. It makes the text clear and at the same time provides an easy availability to experimental details. This idea was used here, that is only the most important information providing the logic of experiments is given in the body text whereas all details are added as supplementary materials for all interested in using these methods or follow the experiments in more details.

ANNEX 13.1. MATERIAL USED IN THE ANALYSIS AND EXPERIMENTAL DESIGN

In total 45 populations were used at different stages of the analyses (Table 13.1.1). These included 6 ecotypes and 11 cultivars of *L. multiflorum*, 14 ecotypes and 12 cultivars of *L. perenne*, and additionally 2 cultivars of *L. hybridum*. All Italian ryegrass ecotypes originated from West and South Europe, while most perennial ryegrass ecotypes were collected in Denmark and Norway and were obtained from the Nordic Gene Bank. The majority of studied cultivars were bred in Europe and they were provided by the Research Centre for Cultivar Testing (COBORU) or Polish breeding stations.

The overall design of field experiments and plant development is presented in Figure 13.1.1. Seeds from all *L. multiflorum* and *L. perenne* populations were germinated in dark at 24°C for six days and then they were transferred onto the glass plates covered with white filter paper in order to estimate the level of fluorescence of seminal roots (Annex 13.2). This test was used to confirm the species identity in addition to morphological characters. Seedling roots of *L. multiflorum* fluoresce when placed under UV light, and those of *L. perenne* do not fluoresce. The plants with faint fluorescence (+/-) were discarded and only plants whose roots had brilliant fluorescence (+) and those that did not show evidence of fluorescence (-) were used in further analyses. Selected seedlings were transplanted to 6.0 by 10.0 cm

plastic pots filled with commercial soil, peat, pH 6.0 and sand by a ratio of 1:1:1. Plants were growing in a growth chamber at a daily temperature of 20°C±2°C with a 12-h daylight period provided by cool white fluorescent light. After two months, in April and early May, as seedlings developed into multitillered plants, each plant was divided into 9-10 ramets and all were transplanted to the experimental field on 2nd class soil. A randomized complete block design with three blocks was used to analyze all populations. Each block consisted of three ramets as replicates of each genotype. Plants were sown randomly in each replication. Plants were spaced on 50 cm by 50 cm grids. The field was situated in the Warmia and Mazury University (UWM) experimental station in Tomaszkowo, five km away from Olsztyn. Granular fertilizer of ammonium sulfate (21-0-0-24, N-P-K-S) was applied at 60 kg/ha before transplantation. The weeds were controlled by interrow application of Chwastox 750 SL herbicide (75% of 4-chloro-2-methylfenoxy acetate) at a rate 0.5 l/ha. Plants were sprayed with Bayleton 5WP (12.5% of triadimefon) at a rate 50l/ha against powdery mildew and rust and with Decis 2.5 EC (2.5% of deltamethrin) at a rate 0.25l/ha against aphids. Plants were cut with a handheld sickle in July and September. All experiments were carried out in 2002 and 2003.

Other *Lolium* species, *Festuca*, Poa

Five *Lolium* species, *Festuca* pratensis and *Poa pratensis* were included in the analysis of phylogenetic relationships within the genus *Lolium*. Seeds originated from gene banks and own resources (Table 13.1.2). The mode of reproduction and ploidy level is given in Table 13.1.3. The methodology of seed germination, the fluorescence test for *Lolium*, plant growing and cultivation were as for *L. multiflorum* and *L. perenne*, except that only one replication experiment was set up in the field.

Representatives of Triticeae and Aveneae

Hordeum vulgare, *Triticum aestivum*, *Secale cereal*e and artificial allopolyploid *Triticale* were included in the analyses as representatives of Triticeae. Among Aveneae, diploid *Avena strigosa* and hexaploid *A. sativa* were included in phylogenetic analyses. Additionally a model species, *Arabidopsis thaliana* as an out-group was used. Seeds were either received from breeding stations or collected by ourselves. All seeds were reproduced for several generations in the experimental field of UWM in Tomaszkowo (Table 13.1.2). Mode of reproduction, life form and ploidy level are given in Table 13.1.3.

F_1 generations and F_2 populations of *L. multiflorum* and *L. perenne*

Several F_1 hybrids and four F_2 populations were used in the genetic mapping (Table 13.1.4). Parental plants were chosen from contrasting populations as estimated by morphological and molecular analyses. Because *L. multiflorum* and *L. perenne* are allogamous species, natural populations as well as cultivars are at least partially heterozygous. Therefore, each potential parent was self-pollinated in 1999 to reduce heterozygosity. In the early spring 2000, seeds were germinated, checked for fluorescence and seedlings were transfer

individually to pots. After about two months, multitillered plants were transplanted to the field in Tomaszkowo and used for crossing experiments. In total 50 plants from five populations of *L. multiflorum* and five populations of *L. perenne* were used.

Each F_1 hybrid was derived from a cross between a single female parent and a single male parent. About ten different crosses were made in 2000. The female parent was chosen to recognize hybrids from accidental self-pollination. Inflorescences of a female plant were emasculated, and pollinated with pollen of a single male plant. All pollinated spikes were isolated with a paper bag. When matured, F_1 seeds were collected, and germinated at 24°C, checked for fluorescence, and F_1 plants were transplanted into a greenhouse during the winter 2000/2001. In total eight F_1 hybrids derived from interspecific crosses (from four different combinations), five hybrids of *L. multiflorum* genotypes (from two combinations) and four hybrids of *L. perenne* genotypes (from two combinations) were grown.

Each F_2 population was derived from self-pollination of a single F_1 hybrid plant so as to ensure 3:1 segregation of dominant markers or 1:2:1 in a case of codominant ones. To prevent from out-pollination and force to self-pollination, each F_1 plant was growing in a different box with cereals or *Poa*. Additionally each F_1 plant was isolated with a paper bag. Seeds were harvested from individual plants. In total from 50 to 400 seeds of F_2 were collected per a single F_1 hybrid. Among interspecific crosses two biggest populations were chosen for further studies. Among intraspecific crosses a population per a species was selected on the basis of the number of individuals. Seeds of these populations were germinated in the laboratory in the autumn 2001 and transplanted into the field in the spring 2002.

The methodology of F_2 seed germination, the fluorescence test, plant growing and cultivation were nearly the same as for *L. multiflorum* and *L. perenne* populations. The modifications were related with the recovery of plants with faint fluorescence (+/-) and a greater number of replications. Each F_1 and F_2 plant and parental plants were divided into 27 ramets. In total three blocks located within Tomaszkowo were set up. Each block consisted of three replications and each replication consisted of three ramets as replicates of each genotype. Hence, in each block a single genotype was represented by nine ramets. Quantitative characters were analysed during two seasons (2002 and 2003) and during two crops (July, September) to assess genotype x environment interaction.

Table 13.1.1. List of *L. multiflorum* and *L. perenne* populations used at the different stages of analyses

Species	Name	Cultivar/ Ecotype	Origin	Seed source[1]	Type of analysis				
					M	E	D	F	C
L. multiflorum	Barball	Ecotype	Italy	Lodi					
	Guliamo		Italy	Lodi					
	German		Germany	Lodi					
	Italian		Italy	Barenburg					
	Spanish		Spain	Barenburg					
	Variamo		Italy	Lodi					
	Atalja	Cultivar	Denmark	COBORU					
	Asso		Italy	COBORU					
	Bartissimo		Italy	COBORU					
	Bartolini		Italy	COBORU					
	Crema		Italy	COBORU					
	Limulta		-	COBORU					
	Ligrande		Germany	COBORU					
	Miyukiaoba		Japan	Japan					
	Nagamamikari		Japan	Japan					
	Tenor		-	COBORU					
	Waseaoba		Japan	Japan					
L. perenne	NGB4262	Ecotype	Norway	Plateforsk, NGB					
	NGB4264		Norway	Plateforsk, NGB					
	NGB5030		Denmark	DLF Trifolium A/S, NGB					
	NGB5031		Denmark	DLF Trifolium A/S, NGB					
	NGB5036		Denmark	DLF Trifolium A/S, NGB					
	NGB10793		Denmark	DLF Trifolium A/S, NGB					
	NGB10795		Denmark	DLF Trifolium A/S, NGB					
	NGB10809		Denmark	DLF Trifolium A/S, NGB					
	NGB10815		Denmark	DLF Trifolium A/S, NGB					
	704		Giełgoń, Poland,	Bydgoszcz					
	708		Kąty, Poland	Bydgoszcz					
	Tatras		The Tatras, Poland	DG					
	Hungarian		Hungary	Barenburg					
	New Zealand		New Zealand	Barenburg					
	Aberoscar	Cultivar	-	Marchwacz					
	Argona		Poland	Marchwacz					
	Ba012		Poland	Bartążek					
	Kyosato		Japan	Marchwacz					
	Lisuna		Germany	COBORU					
	Magella		Netherlands	COBORU					
	Merganda		Belgium	COBORU					
	Numan		USA	Marchwacz					
	Pamir		-	COBORU					
	Portstewart		-	COBORU					
	Rela		Poland	Bartążek					
	Solen		Poland	COBORU					
L. hybridum	Agata	Cultivar	Poland	Marchwacz					
	Gosia		Poland	Marchwacz					

M – morphological variation (Chapter 3), **E –** enzymatic analyses (Chapter 4), **D –** DNA analyses (Chapter 4), **F –** seedling root fluorescence estimation (Chapter 5), **C –** correlation between seedling root fluorescence and morphological characters (Chapter 5)

[1]**Abbreviations used in the text:**

Lodi - Italy
Barenburg - Barenburg Research, Barenburg, Netherlands
COBORU - Research Centre for Cultivar Testing in Słupia Wielka, Poland
Japan - Hokuriku National Experiment Station, Japan
NGB - Nordic Gene Bank, Alnarp, Sweden
Bydgoszcz - Botanical Garden, Bydgoszcz, Poland
DG - Department of Genetics, UWM, Poland. Collected by K. Polok and R. Zielinski
Marchwacz - Breeding Station in Marchwacz, Poland
Bartążek - Breeding Station in Bartążek, Poland

Table 13.1.2. List of species used in phylogenetic studies

Species	Accession details/ cultivar name	Origin	Seed donor	Sampling details
Lolium loliaceum	ABY-BA-13644	France Botanic Garden, Guyancourt	Institute of Grassland and Environmental Research. Genetic Resources Unit. Aberystwyth. Wales. UK.	About 100 seeds of each sample were reproduced in the experimental field in Tomaszkowo (UWM) in 2002
Lolium persicum	ABY-BA-11133	Botanic Garden, Yerevan		
Lolium remotum	ABY-BA-10350	Botanic Garden, Uppsala		
Lolium rigidum	ABY-BA-11918	Italy		
Lolium temulentum	ABY-BA-13141	Setubal, NE of Alcacer		
	NGB6915.2	no data	Nordic Gene Bank, Sweden	
Festuca pratensis	BA592/04	Poland	Institute of Plant Breeding and Acclimatisation. Breeding Station Bartążek, Poland	6 clones were obtained
Poa pratensis		Poland		
Hordeum vulgare	Brenda	Germany	Polish Breeders Ltd, Poznań, Poland	Samples were reproduced in the experimental field in Tomaszkowo (UWM)
Triticum aestivum	ETA	Poland	Breeding station, Kobierzyce, Poland	
Secale cereale	Dańkowskie Złote	Poland	Experimental station UWM in Tomaszkowo, Poland	
Triticale	Kargo	Poland	Experimental station UWM in Tomaszkowo, Poland	
Avena sativa	Collected by Tadeusz Korniak in north-east Poland	Poland	Department of Botany and Nature Protection, UWM, Poland	
Avena strigosa		Poland		
Arabidopsis thaliana	Collected by Kornelia Polok in Olsztyn	Poland	Department of Genetics UWM, Poland	Samples were reproduced in the growth chamber of the Department of Genetics

Table 13.1.3. Main biological characters of studied species

Species	Reproductive system	Life form	N° of chromosomes[1]	Ploidy
Lolium loliaceum	autogamous	annual	14	2n=2x
Lolium persicum	autogamous	annual	14	2n=2x
Lolium remotum	autogamous	annual	14	2n=2x
Lolium temulentum	autogamous	annual	14	2n=2x
Lolium multiflorum	allogamous	annual/biennial	14	2n=2x
Lolium perenne	allogamous	perennial	14	2n=2x
Lolium rigidum	allogamous	annual	14	2n=2x
Festuca pratensis	allogamous	perennial	14	2n=2x
Poa pratensis	allogamous	perennial	56	2n=8x
Hordeum vulgare	autogamous	annual	14	2n=2x
Triticum aestivum	autogamous	annual	42	2n=6x
Secale cereale	allogamous	annual	14	2n=2x
Triticale	allogamous	annual	42	2n=6x
Avena sativa	autogamous	annual	42	2n=6x
Avena strigosa	autogamous	annual	14	2n=2x
Arabidopsis thaliana	autogamous	annual	10	2n=2x(?4x)[2]

[1]Estimated by Katarzyna Kubiak, M.Sc
[2]The newest data suggest that *A. thaliana* is an ancient tetraploid.

Table 13.1.4. List of F_1 and F_2 populations

Abbreviation	Type of a cross	Female parent	Male parent	N° of F_2 individuals
BR3 x NZ15	***L. multiflorum* x *L. perenne***	Bartolini, *L. multiflorum* cultivar, plant N°3	*L. perenne* ecotype from New Zealand, plant N°15	269
HU5 x BO2	***L. perenne* x *L. multiflorum***	*L. perenne* ecotype from Hungary, plant N°5	Bartissimo, *L. multiflorum* cultivar, plant N°2	201
VA7 x AS17	***L. multiflorum* x *L. multiflorum***	Variamo, *L. multiflorum* ecotype, plant N°7	Asso, *L. multiflorum* cultivar, plant N°17	205
KY20 x BB6	***L. perenne* x *L. perenne***	Kyosato, *L. perenne* cultivar, plant N°20	Ba012, *L. perenne* line, plant N°6	44

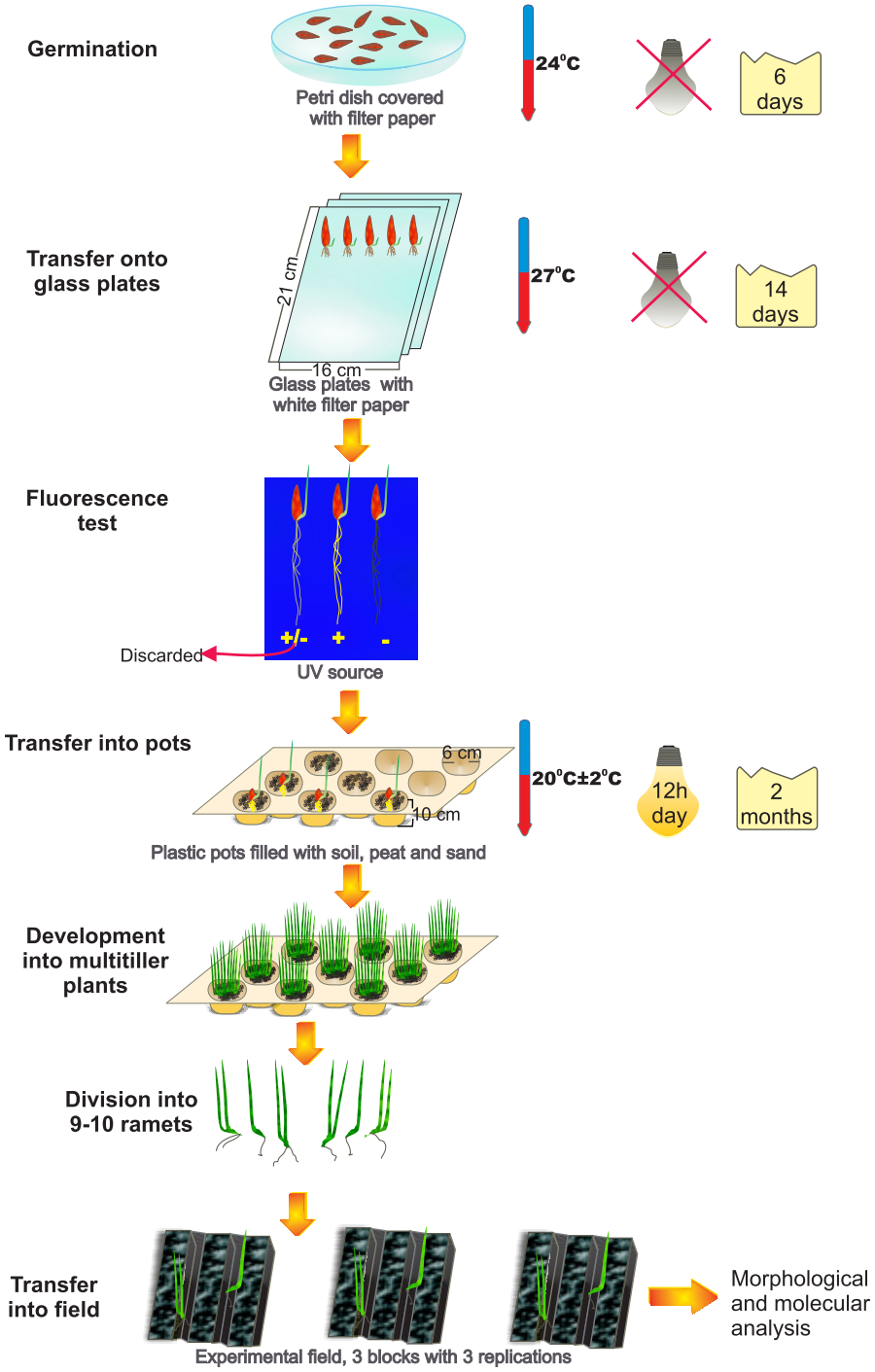


Figure 13.1.1. Material development and field experiments

ANNEX 13.2. SEEDLING ROOT FLUORESCENCE TEST (THE GENTNER'S TEST)

Fluorescence tests were conducted according to Niemyski and Budzyńska (1972) but with own modifications that enabled the standardization of the protocol.

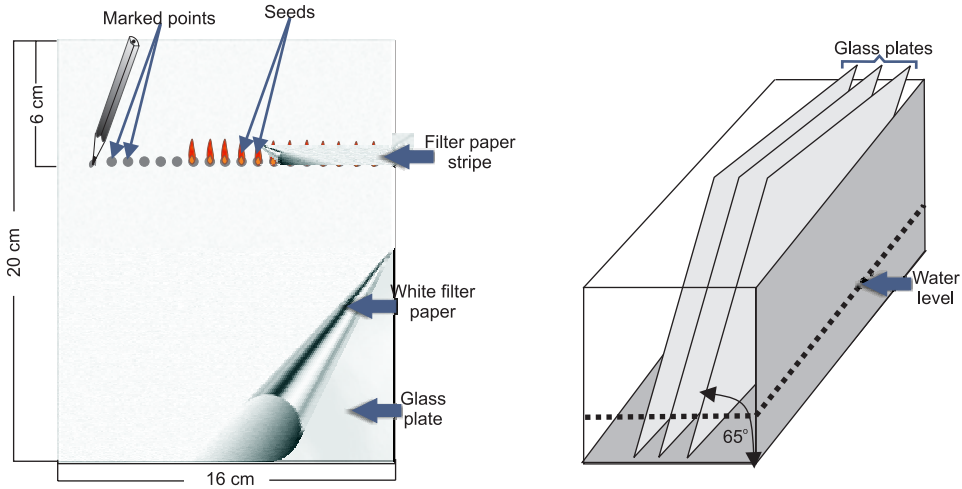
Seed germination

About 30-40 seeds were hand sown in sterile Petri dishes of 10 cm in diameter. White filter paper moistened with tap water was a germination substrate. To keep moisture the internal side of each Petri dish top was covered with wet filter paper. Filter paper humidity was checked daily. Seeds were incubated at 24°C in darkness for six days. Seminal roots of 1-2 mm were visible at this moment. Then roots were checked for fluorescence under UV light. If no fluorescence was visible, seeds were transferred onto glass plates.

Seedling root fluorescence test

Glass plates of 16- by 21 cm were covered with a sheet of white filter paper of 20- by 25 cm. The edges sticking out were folded under the glass plates. Seed places were marked with a pencil at 6-7 cm from the top edge of the glass plate (Figure 13.2.1). Then the filter paper was moistened with tap water.

In total 20 seeds were placed on a glass plate so that the embryos were oriented into the bottom. Then seeds were protected from falling dawn with a wet filter paper stripe of 2.0- by 25 cm (Figure 13.2.1A). Maximum eight glass plates were laid in glass boxes to give a tilt 65° relative to the bottom. This enabled the roots to go down and adhere tight to the filter paper. The last glass plate was covered with an empty glass plate to ensure the comparable conditions for all seeds. Glass boxes were filled with tap water up to 1/4 of the volume (Figure 13.2.1B). The level of water was kept at the same level throughout the whole experiment. The seeds were incubated at 27°C for 14 days. The fluorescence was determined under UV light on the 14th day.



A. Glass plate

B. Glass box with glass plates

Figure 13.2.1. Seedling root fluorescence test

ANNEX 13.3. ISOENZYME ANALYSIS

In total 17 enzyme systems were used in the analysis of genetic diversity and genetic mapping. The enzyme names and numbers used are those recommended by Enzyme Commission (EC), the International Union of Biochemistry (Murphy et al. 1996) and Protein Information Resource (PIR) at http://pir.georgetown.edu (Table 13.3.1).

Table 13.3.1. Enzyme systems used

Enzyme	EC number	Abbreviation	Functional role	Number of loci	Number of alleles
Acid phosphatase	EC 3.1.3.2.	ACP	Hydrolysis of ester bonds in slightly acid pH	**1:** *Acp3*	1
Aconitase hydratase (formerly Aconitase)	EC 4.2.1.3.	ACOH (formerly ACO)	Hydration of cis-aconitate to citrate and isocitrate, tricarboxylic acid cycle	**1:** *Acoh1*	2
Alcohol dehydrogenase	EC 1.1.1.1.	ADH	Zinc protein. Acts on primary or secondary alcohols and on cyclic secondary alcohols in the presence of NAD	**1:** *Adh1*	1
Aspartate transaminase	EC 2.6.1.1.	AAT	It liberates nitrogen from amino acids thus leading to keto-acids.	**3:** *Aat1, Aat2, Aat3*	9
Cytosol aminopeptidase (formerly leucine aminopeptidase)	EC 3.4.11.1.	CAP (formerly LAP)	It is a zinc depended exopeptidase belonging to Peptidase Family M17. It selectively releases N-terminal amino acids residues from polypeptides and proteins. It is involved in the processing, catabolism and degradation of intracellular proteins.	**2:** *Cap1, Cap2*	3
Esterase	EC 3.1.1.-.	EST EST-flu	Carboxylic ester hydrolases. acting on ester bonds.	**8:** *Est1, Est2, Est3, Est4, Est-flu1, Est-flu2, Est-flu3, Est-flu4*	17
Glucose-6-phoshate isomerase (formerly phosphoglucoisomerase)	EC 5.3.1.9.	GPI (formerly PGI)	It is intramolecular oxidoreductase interconverting aldoses and ketoses. Glycolysis. Pentose phosphate pathway and starch and sucrose metabolism.	**1:** *Gpi1*	1
Glutamate dehydrogenase	EC 1.4.1.2.	GTDH	Oxidoreductase acting on the CH-NH_2 group of donors with NAD+ or NADP+ as acceptor. Nitrogen metabolism.	**2:** *Gtdh1, Gtdh2*	3
Hexokinase	EC 2.7.1.1.	HK	Phosphorylation of carbohydrates such as D-fructose, 5-keto-D-fructose, D-glucose, D-mannose etc. Transferring phosphorus-containing groups.	**1:** *Hk1*	2
Isocitrate dehydrogenase	EC 1.1.1.42.	IDH	Acting on the CH-OH group of donors with NADP+ as acceptor. Found in mitochondria and cytoplasm.	**1:** *Idh1*	2
Malate dehydrogenase	EC 1.1.1.37.	MDH	Conversion of L-malate and oxaloacetate using NAD(NADP) as a coenzyme. Also oxidizes some other 2-hydroxydicarboxylic acids.	**2:** *Mdh1, Mdh2*	5
Malate dehydrogenase	EC 1.1.1.40.	MDHP		**2:** *Mdhp1, Mdhp2*	3
Peroxidases	EC 1.11.1.7.	PER	Hemoprotein that catalyses the oxidation of ascorbate, ferrocyanide, cytochrome C and leuco forms of many dyes by hydrogen peroxide	**2:** *Per1, Per2, Per3*	10
Phosphoglucomutase	EC 5.4.2.2.	PGM	Transfer of a phosphate residue from the enzyme to the substrate. The interconversion of 1-phosphate and 6-phosphate isomers and of α-D-ribose 1-phosphate and 5-phosphate.	**1:** *Pgm1*	1
Phosphogluconate dehydrogenase (formerly 6-phosphogluconate dehydrogenase)	EC 1.1.1.44.	PGDH (formerly 6PGD)	Acting on the CH-OH group of donors with NAD+ or NADP+ as acceptor.	**1:** *Pgdh1*	2
Shikimate dehydrogenase	EC 1.1.1.25.	SKDH	Acting on the CH-OH group of donors with NAD+ or NADP+ as acceptor. Phenylalanine, tyrosine and tryptophan biosynthesis	**2:** *Skdh1, Skdh2*	3
Superoxide dismutase	EC 1.15.1.1.	SOD	Destruction of the O_2- free radical, which are normally produced within the cells and which are toxic to biological systems	**2:** *Sod1, Sod2*	2

Preparation of plant material

Approximately 1-2 leaves and a pinch of purified silica sand were put into plexi mortars. For all enzymes but peroxidases, two drops of 0.35% 2-mercaptoethanol were added as the extraction buffer. In a case of peroxidases deionized H_2O was used. The plant material was ground into fine, homogenous and viscous powder with a glass pestle. Wicks of 5 mm by 10 mm were soaked in the leaf extract and protected from drying by covering with plant material. The material was kept at 4°C until use (approximately 15-20 minutes). Then soaked wicks were transferred into the narrow slit in the gel.

Electrophoresis conditions

Lithium borate/Tris citrate buffer system and 11% starch gel were used. Electrophoresis was run at 4°C at constant power up to the moment the front had reached 8 cm from the origin (Table 13.3.2). Once the electrophoresis had been completed, the gel was chilled and then cut into 5-6 slices depending on number of staining enzymes. Each slice was placed in a different stain box and was stained for a single enzyme.

Table 13.3.2. Buffers and electrophoresis conditions

BUFFERS			ELECTROPHORESIS CONDITIONS		
Reagent	Concentration in running buffer	Concentration in gel buffer	Voltage	Amperage	Time of electrophoresis
LiOH x H_2O	0.028 M	0.0028 M	250 V	30-40 mA	½ h
H_3BO_3	0.192 M	0.0192 M	300 V	30-60 mA	½ h
TRIS	-	0.046 M	80 V	10 mA	20 h (overnight)
Citric acid	-	0.080 M	300 V	30-60 mA	1 h
pH	8.1	8.1			

Staining protocols

The distance of migration of specific proteins through the starch gel was visualized by histochemical staining (Table 13.3.3). The own modifications of protocols described by Zielinski (1987) were adopted. Each stain consisted of a substrate, on which a specific enzyme reacts, and dyes or substances that fluoresce under UV light. Some stains also contained cofactors, coupling enzymes and other molecules. All enzymes were stained in 100 ml of staining solution because this technique gave clearer and stronger bands than an agar overlay method. In addition the bands were durable and the gels could be stored without loss of quality in a refrigerator for a long period. All stains but peroxidases were incubated at 37°C until the bands were clearly visible. Peroxidases were stained at room temperature. After staining, the gel was fixed in a methanol, deionized water and glacial acetic acid (60%) by a ratio of 5:5:1 for at least 1 h, covered with plastic food wrap and refrigerated.

Table 13.3.3. Enzyme staining formulas

Enzyme	Reaction	Incubation buffer	Substrate	Dyes	Others
ACP	α-Naphtyl phosphate+H_2O=α-naphtol + P_i α-naphtol + Black K salt **-= diazo-dye**	0.1 M sodium acetate, pH 5.0	•α-naphtyl, 150 mg	•Black K salt, 100 mg	-
AKOH	cis-Aconitate + H_2O = isocitrate isocitrate + NADP = 2-oxaloglutarate + NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•cis-aconitic acid, 100 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 50 mg
ADH	an alcohol + NAD = an aldehyde or ketone + NADH NADH + PMS = NAD + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•ethanol, 6 ml •NAD, 10 mg	•PMS, 2 mg •MTT, 10 mg	-
AAT	L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate oxaloacetate + Fast Blue RR = **diazo-dye**	0.1 M Tris-HCl, pH 8.0	•L-aspartic acid, 150 mg •ketoglutaric acid, 100 mg	•Fast Blue RR, 100 mg	•5-pirydoxal phosphate, 5 mg
CAP	L-leucyl-4-methoxy-2-naphtylamide + H_2O = L-leucinamide + 4-methoxy-2-naphtol 4-methoxy-2-naphtol + Black K salt = **diazo-dye**	0.1 M phosphate, pH 6.0	•L-leucyl-β-naphtylamide HCl, 40 mg	•Black K salt, 50 mg	-
EST	α-Naphtyl acetate (β-Naphtyl acetate) +H_2O=α-naphtol (β-naphtol) + P_i α-naphtol (β-naphtol) + Fast Blue RR **-= diazo-dye**	0.1 M phosphate, pH 6.0	•α-naphtyl acetate, 60 mg •β-naphtyl acetate, 30 mg	•Fast Blue RR, 60 mg	-
EST-flu	4-Methylumbelliferyl acetate + H_2O = acetate + **4-methylumbelliferone**	0.1 M phosphate, pH 6.0	•4-methyl-umbellipheryl acetate, 4 mg	-	-
GPI	D-Fructose-6-phosphate = D-glucose-6-phosphate D-glucose-6-phosphate + NADP (+glucose-6-phosphate dehydrogenase) = D-glucono-δ-lactone-6-phosphate + NADPH NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•D-Fructoso-6-phosphate, 50 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 50 mg •Glucoso-6-phosphate dehydro-genase, 20U
GTDH	L-Glutamate + H_2O + NAD = L-oxoglutarate + NH_3 + NADH NADH + PMS = NAD + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•Glutamic acid, 1-2 g •NAD, 10 mg	•PMS, 2 mg •MTT, 10 mg	-
HK	ATP + α-D-glucose = ADP + glucose-6-phosphate glucose-6-phosphate + NADP (+glucose-6-phosphate dehydrogenase) = 6-phosphogluconate + NADPH NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•Glucose, 50 mg •ATP, 25 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 50 mg •Glucoso-6-phosphate dehydro-genase, 20U
IDH	D-Isocitrate + NADP = 2-oxoglutarate + CO_2 + NADPH NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•DL isocitric acid, 100 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 50 mg
MDH	L-Malate + NAD = oxaloacetate + NADH NADH + PMS = NAD + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 7.2	•Malic acid, 400 mg •NAD, 10 mg	•PMS, 2 mg •MTT, 10 mg	
MDHP	L-Malate + NADP = pyruvate + NADPH NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 7.2	•Malic acid, 400 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 50 mg
PER	H_2O_2 + Amino-ethylcarbazole = H2O + **oxidized 9-amino-ethylcarbazole**	0.05 M sodium acetate, pH 5.0	•H_2O_2, 0.03%	•3-amino-9-ethylcarbasole, 100 mg	•$MgCl_2$ x $6H_2O$, 0.01%
PGM	α-D-Glucose-1,6-biphosphate + α-D-glucose-1-phosphate = α-D-glucose-6-phosphate + α-D-glucose-1,6-biphosphate α-D-glucose-6-phosphate + NADP (+glucose-6-phosphate dehydrogenase) = 6-phosphogluconate + NADPH NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•glucoso-1-phosphate Na_2 salt x $4H_2O$, 150 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 50 mg •Glucoso-6-phosphate dehydro-genase, 20U
PGDH	6-Phospho-D-gluconate + NADP = D-ribuloso-5-phosphate + CO_2 + NADPH NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•6-phospho-gluconic acid Ba salt, 40 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 0.01%
SKDH	Shikimic acid + NADP = ...+ NADPH NADPH + PMS = NADP + reduced PMS reduced PMS + MTT = PMS + **formazane**	0.1 M Tris-HCl, pH 8.0	•shikimic acid Ba salt, 30 mg •NADP, 10 mg	•PMS, 2 mg •MTT, 10 mg	•$MgCl_2$ x $6H_2O$, 0.01%
SOD	MTT + riboflavin + EDTA = reduced MTT reduced MTT + O_{2^-} = **MTT colourless on blue background**	0.05 M Tris-HCl, ph 8.2	•EDTA, 75 mg	•MTT, 10 mg	•Riboflavin, 3 mg

Bold characters indicate colour products

ANNEX 13.4. DNA ISOLATION

A modified version of CTAB method (Murray and Thompson 1980) was used for DNA isolation. In this procedure nucleic acids form soluble complexes with a detergent CTAB (Cetyltrimethylammonium bromide) in the presence of high concentration of NaCl. The complex nucleic acids-CTAB is extracted from plant material with a chloroform and isoamyl alcohol mixture. Polysaccharides, phenols and the others are removed by the precipitation of the nucleic acid-CTAB complex in environment of low salt concentration. In these conditions the complexes CTAB-nucleic acids form pellets while contaminants stay in a supernatant. CTAB is removed from pellets by dissolving the CTAB-nucleic acid complex in a high concentration of salt (NaCl or CsCl) and precipitation of nucleic acids with ethanol or isopropanol.

The leaves for DNA isolation were collected from plants growing in the field approximately a month after the autumn cut in 2002 and 2003. About 4-5 g of leaves per a plant was packed in a plastic, marked bag, sealed and kept in a cool place up to the moment they were transferred to the laboratory. Then they were frozen and stored in -30°C until use. DNA was isolated from about 1 g of leaves. Prior to DNA isolation, all leaves were washed in deionized water and surface sterilized with 96% ethanol. Then they were ground into a fine powder in liquid nitrogen and thoroughly mixed with 3 ml of pre-warm CTAB isolation buffer (Table 13.4.1). The mixture was incubated for 60 min. at 55°C with occasional shaking. Afterwards, nucleic acids were extracted three times with chloroform and isoamyl alcohol by a ratio 24:1, precipitated with the CTAB precipitation buffer (Table 13.4.1) for 24 h at room temperature and dissolved in 1 ml of 1 M cesium chloride for 30 min. at 37°C. After the subsequent precipitation with 2.5 volume of ethanol, RNA was removed with RNA-ase at a final concentration 200 g/ml (Table 13.4.2). The DNA was then precipitated with 96% ethanol and the pellet was washed with 70% ethanol and dissolved in deionized water. The quality of DNA was verified on 1% (w/v) agarose containing 0.5 g/ml ethidium bromide and separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power. The purity of DNA was assessed spectrophotometrically and averaged 95-98%. Finally the aliquots were prepared at a final concentration 500, 750 or 1000 ng/l. The DNA was stored at -30°C until use.

Table 13.4.1. Buffers used for DNA isolation

Reagent	Concentration	
	CTAB isolation buffer	CTAB precipitation buffer
CTAB (Cetyltrimethylammonium bromide)	2% (m/v)	1% (m/v)
1 M Tris-HCl, pH 8.0	100 mM	50 mM
0.5 M EDTA	20 mM	10 mM
5 M NaCl	1.4 M	-
2-Mercaptoethanol (just before use)	2% (v/v)	2% (v/v)

Table 13.4.2. Reaction buffer for RNA-ase

Reagent	Concentration
1 M Tris-HCl, pH 7.5	10 mM
5 M NaCl	15 mM
10 mg of RNA-ase was dissolved in 1 ml of a buffer, boiled for 15 min and stored at -20°C	

ANNEX 13.5. LIST OF PRIMERS USED IN THE STUDIES

Abbreviation	Sequence 5'→3'	Method	Application			
			Genetic diversity	Fluores-cence	Mapping	Phylo-genesis
Amplification of organelle genomes						
psbC-trnS-F	5'-GGT CGT GAC CAA GAA ACC A-C3'	Amplification of non-coding region flanking by *psbC* and *trnS* genes in chloroplast DNA	■			■
psbC-trnS-R	5'-GGT TCG AAT CCC TCT CTC TC-3'					
nad1-F	5'-GCA TTA CGA TCT GCA GCT CA-3'	Amplification of intron between nad1b and nad1C exons in *nad1* gene of mitochondrial DNA	■			■
nad1-R	5'-GGA GCT CGA TTA GTT TCT GC-3'					
LOLMTI-F	5'-CTC TTT ACT TTT ACT GCC TGT GC-3'	Amplification of mitochondrial gene *LOLMTI* homologous to chloroplast *rps19*				■
LOLMTI-R	5'-GCT CGC CTT TCG GGG TGA CGC-3'					
Random Amplified Polymorphic DNA (RAPD)						
KP01	5'-AGC CTG ATG C-3'	RAPD, primers designed by ourselves	■			
KP02	5'-TCT GTC ATC G-3'		■			
KP03	5'-AAC ATG CTG G-3'		■			
KP04	5'-AAA GTA GCG C-3'		■			
KP05	5'-AGC CAG GTA G-3'		■			
KP06	5'-CTG CTT GGG T-3'		■			
KP07	5'-ACG GAT CGC G-3'		■			
OPA01	5'-CAG GCC CTT C-3'	RAPD, primers from Operon Technologies		■	■	
OPA02	5'-TGC CGA GCT G-3'		■	■	■	
OPA03	5'-AGT CAG CCA C-3'		■	■	■	
OPA04	5'-AAT CGG GCT G-3'				■	
OPA05	5'-AGG GGT CTT G-3'				■	
OPA06	5'-GGT CCC TGA C-3'			■	■	
OPA07	5'-GAA ACG GGT G-3'			■	■	
OPA08	5'-GTG ACG TAG G-3'			■	■	■
OPA09	5'-GGG TAA CGC C-3'			■	■	
OPA10	5'-GTG ATC GCA G-3'			■	■	
OPA11	5'-CAA TCG CCG T-3'			■	■	
OPA12	5'-TCG GCG ATA G-3'			■	■	■
OPA13	5'-CAG CAC CCA C-3'			■	■	
OPA14	5'-TCT GTG CTG G-3'			■	■	
OPA15	5'-TTC CGA ACC C-3'				■	
OPA16	5'-AGC CAG CGA A-3'				■	
OPA17	5'-GAC CGC TTG T-3'				■	
OPA18	5'-AGG TGA CCG T-3'				■	
OPA19	5'-CAA ACG TCG G-3'			■	■	■
OPA20	5'-GTT GCG ATC C-3'			■	■	■
OPB01	5'-GTT TCG CTC C-3'			■	■	■
OPB02	5'-TGA TCC CTG G-3'			■	■	
OPB03	5'-CAT CCC CCT G-3'			■	■	■
OPB04	5'-GGA CTG GAG T-3'			■	■	
OPB05	5'-TGC GCC CTT G-3'			■	■	
OPB06	5'-TGC TCT GCC C-3'			■	■	
OPB07	5'-GGT GAC GCA G-3'			■	■	
OPB08	5'-GTC CAC ACG G-3'			■	■	
OPB09	5'-TGG GGG ACT C-3'			■	■	

ANNEX 13.5. LIST OF PRIMERS, CONTINUED

Abbreviation	Sequence 5'→3'	Method	Application			
			Genetic diversity	Fluores-cence	Mapping	Phylo-genesis
OPB10	5'-CTG CTG GGA C-3'	RAPD, primers from Operon Technologies		■	■	
OPB11	5'-GTA GAC CCG T-3'			■	■	
OPB12	5'-CCT TGA CGC A-3'			■	■	
OPB13	5'-TTC CCC CGC T-3'			■	■	■
OPB14	5'-TCC GCT CTG G-3'			■	■	
OPB15	5'-GGA GGG TGT T-3'			■	■	
OPB16	5'-TTT GCC CGG A-3'			■	■	■
OPB17	5'-AGG GAA CGA G-3'			■	■	
OPB18	5'-CCA CAG CAG T-3'			■	■	
OPB19	5'-ACC CCC GAA G-3'			■	■	■
OPB20	5'-GGA CCC TTA C-3'			■	■	■
OPD01	5'-ACC GCG AAG G-3'			■	■	
OPD02	5'-GGA CCC AAC C-3'			■	■	
OPD03	5'-GTC GCC GTC A-3'			■	■	
OPD04	5'-TCT GGT GAG G-3'			■	■	
OPD05	5'-TGA GCG GAC A-3'			■	■	
OPD06	5'-ACC TGA ACG G-3'			■	■	
OPD07	5'-TTG GCA CGG G-3'			■	■	
OPD08	5'-GTG TGC CCC A-3'			■	■	
OPD09	5'-CTC TGG AGA C-3'			■	■	
OPD10	5'-GGT CTA CAC C-3'			■	■	
OPD11	5'-AGC GCC ATT G-3'			■	■	
OPD12	5'-CAC CGT ATC C-3'			■	■	
OPD13	5'-GGG GTG ACG A-3'			■	■	
OPD14	5'-CTT CCC CAA G-3'			■	■	
OPD15	5'-CAT CCG TGC T-3'			■	■	
OPD16	5'-AGG GCG TAA G-3'			■	■	
OPD17	5'-TTT CCC ACG G-3'			■	■	■
OPD18	5'-GAG AGC CAA C-3'			■	■	
OPD19	5'-CTG GGG ACT T-3'			■	■	■
OPD20	5'-ACC CGG TCA C-3'			■	■	
Semi-specific markers – Intron Splice Junction markers (ISJ)						
ISJ1	5'-CAG ACC TGC A-3'	ISJ primers designed by ourselves based on consensus sequences at the intron splice junctions	■		■	■
ISJ2	5'-ACT TAC CTG AGG CGC CAC-3'		■	■	■	■
ISJ3	5'-TGC AGG TCA G-3'		■	■	■	■
ISJ4	5'-GTC GGC GGA CAG GTA AGT-3'		■	■	■	■
ISJ5	5'-CAG GGT CCC ACC TGC A-3'				■	■
ISJ6	5'-ACT TAC CTG AGC CAG CGA-3'				■	■
ISJ7	5'-TGC AGG TCA GGA CCC T-3'				■	■
ISJ8	5'-GAC CGC TTG CAG GTA AGT-3'				■	■
ISJ9	5'-AGG TGA CCG ACC TGC A-3'				■	■
ISJ10	5'-ACT TAC CTG CAT CCC CCT-3'				■	■
ISJ11	5'-TGC AGG TCA AAC GTC G-3'				■	■
ISJ12	5'-GGA CTG GAG CAG GTA AGT-3'				■	■

ANNEX 13.5. LIST OF PRIMERS, CONTINUED

Abbreviation	Sequence 5'→3'	Method	Application			
			Genetic diversity	Fluores-cence	Mapping	Phylo-genesis
Simple Sequence Repeats - microsatellites (SSR)						
A02-F	5'-AAA GAC CGC ATA CGA AGT-3'	Simple Sequence Repeats (SSR) derived from *L. perenne* SSR-enriched library by Jones et al. (2001). Primers showing high polymorphism and cross species amplification were selected.				
A02-R	5'-AAC CAA AGC CTC AAG ACA-3'					
A07-F	5'-TGG AGG GCT CGT GGA GAA GT-3'					
A07-R	5'-CGG TTC CCA CGC CTT GC-3'					
A10-F	5'-GAG GCA CCG GCC ATG GAG-3'					
A10-R	5'-AGG ACG AGC CAC TCA CTT G-3'					
A11-F	5'-CGG CCA CCC TTG ATA GAG-3'					
A11-R	5'-TCG TCA AGG ATC CGG AGA-3'					
C11-F	5'-TGG AATAAC GAT GAA AAG-3'					
C11-R	5'-CAT CAC GAA TTA ACA AGA G-3'					
E10-F	5'-CGC AGC TTA ATT TAG TC-3'					
E10-R	5'-GCT TTG AGT ATG TAA AGT T-3'					
H06-F	5'-ATT GAC TGG CTT CCG TGT T-3'					
H06-R	5'-CGC GAT TGC AGA TTC TTG-3'					
Amplified Fragment Length Polymorphism primers (AFLP) Sequence Specific Amplification Polymorphism - universal primers (SSAP)						
EcoAd-F	5'-CTC GTA GAC TGC GTA CC-3'	AFLP adapters compatible with an *Eco*RI restriction site				
EcoAd-R	5'-AAT TGG TAC GCA GTC TAC-3'					
PstAd-F	5'-CTC GTA GAC TGC GTA CAT GCA-3'	SSAP adapters compatible with an *Pst*I restriction site				
PstAd-R	5'-TGT ACG CAG TCT AC-3'					
MseAd-F	5'-GAC GAT GAG TCC TGA G-3'	AFLP and SSAP adapters compatible with an *Mse*I restriction site				
MseAd-R	5'-TAC TCA GGA CTC AT-3'					
Pst0	5'-GAC TGC GTA CAT GCA G-3'	SSAP pre-amplification primer complementary to PstAd annealed adapters				
Mse0	5'-GAT GAG TCC TGA GTA A-3'	SSAP pre-amplification primer complementary to MseAd annealed adapters				
EcoRI-A	5'-GAC TGC GTA CCA ATT C**A**-3'	AFLP pre-amplification primer complementary to EcoAd annealed adapters with a selective base, A				
MseC	5'-GAT GAG TCC TGA GTA A**C**-3'	AFLP pre-amplification primer complementary to MseAd annealed adapters with a selective base, C				
Mse-CT	5'-GAT GAG TCC TGA GTA A**CT**-3'	SSAP selective amplification primers complementary to MseAd annealed adapters with two or three selective bases				
Mse-CC	5'-GAT GAG TCC TGA GTA A**CC**-3'					
Mse-ACA	5'-GAT GAG TCC TGA GTA A**AC A**-3'					
Mse-CAA	5'-GAT GAG TCC TGA GTA A**CA A**-3'	AFLP selective amplification primers complementary to MseAd annealed adapters with three selective bases				
Mse-CAC	5'-GAT GAG TCC TGA GTA A**CA C**-3'					
Mse-CAT	5'-GAT GAG TCC TGA GTA A**CA T**-3'					
Mse-CTC	5'-GAT GAG TCC TGA GTA A**CT C**-3'					
Mse-CTG	5'-GAT GAG TCC TGA GTA A**CT G**-3'					
Mse-CTT	5'-GAT GAG TCC TGA GTA A**CT T**-3'					

ANNEX 13.5. LIST OF PRIMERS, CONTINUED

Abbreviation	Sequence 5'→3'	Method	Application			
			Genetic diversity	Fluores-cence	Mapping	Phylo-genesis
EcoRI-AAC	5'-GAC TGC GTA CCA ATT C**AA C**-3'	AFLP selective amplification primer complementary to EcoAd annealed adapters with three selective bases				
EcoRI-AAT	5'-GAC TGC GTA CCA ATT C**AA T**-3'					
EcoRI-ACC	5'-GAC TGC GTA CCA ATT C**AC C**-3'					
EcoRI-ACG	5'-GAC TGC GTA CCA ATT C**AC G**-3'					
EcoRI-ACT	5'-GAC TGC GTA CCA ATT C**AC T**-3'					
EcoRI-AGC	5'-GAC TGC GTA CCA ATT C**AG C**-3'					
Pst-AT	5'-GAC TGC GTA CAT GCA G**AT**-3'	SSAP, selective amplification primers complementary to PstAd annealed adapters with two selective bases				
Sequence Specific Amplification Polymorphism - transposon specific primers (SSAP)						
CACTA	5'-GAT TGC CTT TTT CCT AGT AGT G-3'	amplifies *Tpo1*				
LTR1	5'-GAG CAA GAA TTA CTC TAA CAC-3'	amplifies *Lolcopia1*				
LTR2	5'-ACA GAG ACT TAT CCA TCA C-3'	amplifies *Lolcopia2*				
Bacteria Specific Amplification Polymorphism (B-SAP) katG primers						
katG1-F	5'GAC TAC GCC CAA CAG CTC C3'	Primers complementary to *M. tuberculosis KatG* gene encoding catalase-peroxidase. Primers are designed to target *KatG* every 280 bp				
katG1-R	5'GCG ATA ACC CCG CAA GAC C3'					
katG2-F	5'GCG GGG TTA TCG CCG ATG3'					
katG2-R	5'GCC CTC GAC GGG GTA TTT C3'					
katG3-F	5'AAC GGC TGT CCC GTC GTG3'					
katG3-R	5'GTC GTG GAT GCG GTA GGT G3'					
katG4-F	5'TCG ACT TGA CGC CCT GAC G3'					
katG4-R	5'CAG GTC CGC CCA TGA GAG3'					
katG5-F	5'CGA CAA CGC CAG CTT GGA C3'					
katG5-R	5'GGT TCA CGT AGA TCA GCC CC3'					
katG6-F	5'GCA GAT GGG GCT GAT CTA CG3'					
katG6-R	5'ACC TCG ATG CCG CTG GTG3'					
katG7-F	5'GCT GGA GCA GAT GGG CTT G3'					
katG7-R	5'ATC CAC CCG CAG CGA GAG3'					
katG8-F	5'GTC ACT GAC CTC TCG CTG3'					
katG8-R	5'CGC CCA TGC GGT CGA AAC3'					
katG9-F	5'GCG AAG CAG ATT GCC AGC C3'					
katG9-R	5'ACA GCC ACC GAG CAC GAC3'					
katG10-F	5'CAA AGT GTC CTT CGC CGA CC3'					
katG10-R	5'CAC CTA CCA GCA CCG TCA TC3'					
katG11-F	5'TGC TCG ACA AGG AGA ACC TG3'					
katG11-R	5'TCC GAG TTG GAC CCG AAG AC3'					
katG12-F	5'TAC CAG GGC AAG GAT GGC AG3'					
katG12-R	5'GCA AAC ACC AGC ACC CCG3'					

ANNEX 13.5. LIST OF PRIMERS, CONTINUED

Abbreviation	Sequence 5'→3'	Method	Application			
			Genetic diversity	Fluores-cence	Mapping	Phylo-genesis
Bacteria Specific Amplification Polymorphism (B-SAP)						
Other primers						
IS-1	5'ACC CCA TCC TTT CCA AGA AC3'	Outer primers complementary to *M. tuberculosis* insertion element IS6110				
IS2	5'GGC TGA GGT CTC AGA TCA G3'					
pol-F	5'AGC GGA TGA CCA CCC AGG AC3'	Primers complementary to a 303 bp fragment of *M. tuberculosis pol* gene				
pol-R	5'TAC ACC GAC AGC GAG CCG AT3'					
rpo-F	5'CGC TGA CCG AAG AAG ACG3'	Primers complementary to a 611 bp fragment of *M. tuberculosis rpo* gene				
rpo-R	5'CTC GTC GGC GGT CAG GTA3'					
Sequence-tagged sites (STS)						
LOLPI-F	5'CCT CCT CGT CGG TGC TCC TGG3'	Primers derived from *L. perenne* gene LOLPISO5A encoding pollen allergen Lol p I				
LOLPI-R	5'ACT TGT CCT TGC CCT TCT CCT3'					
LOLP3-F	5'CGC TGT TCG CCG TGT TCC TGG3'	Primers derived from 3' end of *L. perenne* gene LOLPISOIA encoding pollen allergen Lol p I				
LOLP3-R	5'ATT TGA TTG GGT GCC TCC T3'					
LOP1B-F	5'GCC GCT CCT ACG CCG CTG ACG3'	Primers derived from *L. perenne* gene LOLLOPIB encoding pollen allergen Lol p Ib				
LOP1B-R	5'ACC CTC TCG ATC GCG GCT GC3'					
ASNL-F	5'GGC GTG GAC CCG AAC CTG3'	Primers derived from *L. perenne* gene encoding asparagine synthetase				
ASNL-R	5'TGG GTA CCT CAC TCC CAG ATG CCG ACC TC3'					
Trx-F	5'GAC TTA TCC CCA ACT CA3'	Primers derived from *L. perenne* gene encoding thioredoxin				
Trx-R	5'CCA TCG CCA ATA GC3'					
Gln2-F	5'GGG TTG GAG GGT CTG GAA T3'	Primers derived from *L. perenne* gene encoding glutamine synthetase				
Gln2-R	5'TGA CGA AGT GAA AGG TTC AG3'					
LOLITS-F	5'CTG GGC GGC GGC ATC GTC3'	Primers derived from *L. perenne* Internal transcribed region				
LOLITS-R	5'GCC GCG TCC CAC CCC AAG TTA GT3'					
LTLHAB-F	5'AGT CTC CTC CTC CTC CGC TGC CA3'	Primers derived from *L. temulentum* nuclear gene encoding chlorophyll binding protein				
LTLHAB-R	5'CCA TCA TCA CAA TCT ACT CCC CA3'					
ASN-F	5'GCA CCG TCG AGA TGG AGG CCA3'	Primers derived from *L. perenne* gene encoding L-asparaginase				
ASN-R	5'TGG GTA CCT CAC TCC CAG ATG CCG ACC TC3'					
AS1-F	5'ATG ACA GAC GTT CCA TTC GG3'	Primers derived from *L. perenne* gene encoding asparagine synthetase				
AS1-R	5'CCA GAG ATG ACA TCT TGA CC3'					
HS1-F	5'GAC CCG GAC GAT TTC CTG3'	Primers derived from *H. vulgare* gene encoding HS1 protein				
HS1-R	5'GTC TTG GCG GCG TCG AGG3'					
BCD450-F	5'GGT ACA TCA TTC TCA GTC CC3'	Primers derived from *H. vulgare* RFLP probe BCD450				
BCD450-R	5'ACC GGC CTA TGA AAG TTG AGA3'					
CDO504-F	5'ACG ACG ACC CGA AGA G3'	Primers derived from *A. sativa* RFLP probe CDO504				
CDO504-R	5'GCG GTG TGG ACG AGA3'					
CDO1508-F	5'ATG CGG GCG TTG CT3'	Primers derived from *A. sativa* RFLP probe CDO1508				
CDO1508-R	5'GGC GGA TAT GGG AGG TG3'					
ITS-F	5'TCC TCC GCT TAT TGA TAT GC3'	Primers designed to amplify ITS1 region in rDNA				
ITS-R	5'GGA AGT AAA AGT CGT AAC AAG G3'					
tRNA-leu-F	5'CGG AAT TCGGTT CAA ATC CCA CTT CT3'	Primers designed to amplify spacer between leucine tRNA in *Pellia* sp.				
tRNA-leu-R	5'GVT GGA TCC CGC CTT AGA CCA CTC G3'					

ANNEX 13.6. AMPLIFICATION OF CHLOROPLAST DNA (cpDNA)

Total DNA of individual plants was used in PCR reaction. The non-coding region of chloroplast genome bounded by *psbC* (psII 44 KD protein) and *trnS* [tRNA-Ser(UGA)] conservative coding regions was amplified by the pair of "universal primers" with following sequences:

- psbC-trnS-F 5'GGT CGT GAC CAA GAA ACC AC3'
- psbC-trnS-R 5'GGT TCG AAT CCC TCT CTC TC3'

At the first step, the annealing temperature was optimized using PTC200 gradient thermal cycler (BioRad). Each PCR reaction was performed in a total volume 20 µl. Concentrations of reagents and thermal conditions are shown in Table 13.6.1. PCR products (5 µl) were loaded on 1.5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide and separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power. PCR products were digested with three restriction enzymes as follow: *Dra*I, *EcoR*I and *Hae*III. Each reaction mixture of 20 µl contained 5 µl of a PCR product and 3 U of restriction enzyme. Restriction fragments were separated on 2% agarose gels, visualized under UV light (312 nm), photographed and stored as .jpg files. Each restriction fragment was scored as present (1) and absent (0).

Table 13.6.1. Optimum PCR conditions for amplification of *psbC-trnS* fragment

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	**1. Initial denaturation**	94°C	3
•**$MgCl_2$**	2.0 mM	**35 cycles**		
•**10 x Enhancer** containing betaine	1x	**2. Denaturation**	94°C	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 µM	**3. Annealing**	54°C	1
•**psbC-trnS-F primer**	1 µM	**4. Elongation**	72°C	1.30
•**psbC-trnS-R primer**	1 µM	**5. Final elongation**	72°C	10
•**TFL polymerase** (Epicentre Technology)	0.75 U	**6. Soak**	4°C	∞
•**DNA**	80 ng			

ANNEX 13.7. AMPLIFICATION OF MITOCHONDRIAL DNA (mtDNA)

Amplification of the intron between B and C exons in *nad1* gene

Total DNA of individual plants was used in PCR reaction. The intron between B and C exons in *nad1* gene of mitochondrial genome was amplified by the pair of "universal primers" with following sequences:

- nad1-F 5'GCA TTA CGA TCT GCA GCT CA3'
- nad1-R 5'GGA GCT CGA TTA GTT TCT GC3'

At the first step, the annealing temperature between 48°C and 58°C was tested using PTC200 gradient thermal cycler (BioRad). Because no single product was obtained, the temperature with clear reproducible pattern was regarded as optimal. The maternal inheritance of observed bands was verified in F_1 and 20 F_2 individuals derived from a cross between *L. multiflorum* cultivar, Bartolini and *L. perenne* ecotype from New *Zea*land.

Each PCR reaction was performed in a total volume 20 µl. Concentrations of reagents and thermal conditions are shown in Table 13.7.1. PCR products were loaded on 2% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files. Each fragment was scored as present (1) and absent (0).

Table 13.7.1. Optimum PCR conditions for amplification of intron between B and C exons in *nad1* gene

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	**1. Initial denaturation**	94°C	3
•**$MgCl_2$**	2.0 mM	**35 cycles**		
•**10 x Enhancer** containing betaine	1x	**2. Denaturation**	94°C	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 µM	**3. Annealing**	54°C	1
•**nad1-F primer**	1 µM	**4. Elongation**	72°C	1.30
•**nad1-R primer**	1 µM	**5. Final elongation**	72°C	10
•**TFL polymerase** (Epicentre Technology)	0.75 U	**6. Soak**	4°C	∞
•**DNA**	80 ng			

Analysis of *L. multiflorum* mitochondrial gene, LOLMTI (Accession N: D28336)

The *LOLMTI* gene was isolated from *L. multiflorum* mitochondria (Watanabe et al. 1994). The gene is 2 790 bp long and it is homologous to rice chloroplast DNA sequence *rps19*. It is present in mitochondria of many cereals and grasses. The transfer of the chloroplast sequence occurred in the distant past during the evolution of Poaceae. The fragments have been variously rearranged among species with respect to their terminus.

Total DNA of individual plants was used in PCR reaction. The following primers were designed to amplify the whole gene:

- LOLMTI-F 5'CTC TTT ACT TTT ACT GCC TGT GC3'
- LOLMTI-R 5'GCT CGC CTT TCG GGG TGA CGC 3'

At the first step, the annealing temperature between 45°C and 60°C was tested using PTC200 gradient thermal cycler (BioRad) and two samples of each *L. multiflorum* and *L. perenne*. Temperature, at which a product of expected length was received, was considered as the optimal and used in comparative analyses. In addition the concentration of $MgCl_2$ and TFL polymerase was optimized. Finally a single band of 2 790 bp was observed for five species, *L. loliaceum*, *L. multiflorum*, *L. perenne*, *L. rigidum* and *F. pratensis*. The remaining studied species gave multi-bands products. Because all PCR patterns were reproducible the amplification polymorphism was analysed in 16 species. Each fragment was scored as present (1) and absent (0). Furthermore, the restriction analysis was applied for all species revealing a single band.

Each PCR reaction was performed in a total volume 20 μl. Concentrations of reagents and thermal conditions are shown in Table 13.7.2. PCR products were loaded on 2% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files. Where applicable, PCR products were digested with *Alu*I, *Hae*III, *Hind*III, *Hin*fI, *Msp*I and *Taq*I. Shortly, each reaction mixture of 20 μl contained 5 μl of PCR product and 3 U of a restriction enzyme. Restriction fragments were separated on 2% agarose gels. Enzymes were selected based on virtual digestion using NEB cutter.

Table 13.7.2. Optimum PCR conditions for amplification of *LOLMTI* mitochondrial gene

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	**1. Initial denaturation**	94°C	3
•**$MgCl_2$**	2.0 mM	**40 cycles**		
•**10 x Enhancer** containing betaine	1x	**2. Denaturation**	94°C	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 µM	**3. Annealing**	50°C	1
•**nad1-F primer**	1 µM	**4. Elongation**	72°C	2
•**nad1-R primer**	1 µM	**5. Final elongation**	72°C	5
•**TFL polymerase** (Epicentre Technology)	0.75 U	**6. Soak**	4°C	∞
•**DNA**	100 ng			

ANNEX 13.8. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

The system is based on the ability to amplify genomic DNA by a random, 10-nucleotide primer. The amplification product is generated for each genomic region that happens to be flanked by a pair of 10-base primer sites (Figure 13.8.1). A particular DNA fragment, which is generated for one individual but no for the other, represents a DNA polymorphism and can be used as genetic marker. In total 67 primers were used including seven designed by ourselves and 60 from commercial kits (Operon Technologies). Sequences of all primers are given in Annex 13.5. Designed primers were noted using KP prefix and OP prefix was applied for Operon ones. For example, the third sequence from kit A is labelled OPA03. The specific polymorphic products were referred according to Operon recommendations as follow:

- OPA03-1 when referring to a product number counting from anode;
- KP06-2 in a case of designed primers.

The dominant mode of inheritance of markers generated by all primers used in this study was verified in F_1 and F_2 samples derived from four intra- and interspecific *Lolium* crosses. The presence of a band was dominant over the lack of it.

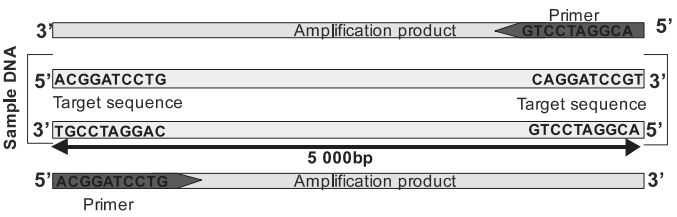


Figure 13.8.1. Principle of RAPD methodology

Each PCR reaction was performed in a total volume 20 µl. Concentrations of reagents and thermal conditions are shown in Table 13.8.1. PCR products were loaded on 1.5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide, separated in 1 x

TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files. All bands that could be reliable read were scored either present (1) or absent (0).

Table 13.8.1. PCR conditions for RAPD markers

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•20 x PCR Buffer 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	1. Initial denaturation	94°C	3
•$MgCl_2$	2.0 mM	46 cycles		
•10 x Enhancer containing betaine	2x	2. Denaturation	94°C	1
•dNTP (dATP, dGTP, dCTP, dTTP)	200 µM	3. Annealing	36°C	1
•10 nucleotide primer	0.3 µM	4. Elongation	72°C	2.30
•TFL polymerase (Epicentre Technology)	1 U	5. Final elongation	72°C	5
•DNA	80 ng	6. Soak	4°C	∞

ANNEX 13.9. INTRON SPLICE JUNCTION MARKERS (ISJ)

The system uses sequences with partial homology to the target sequence to prime the PCR (Weining and Landridge 1991). The sequences of ISJ primers are based on the consensus sequences for the intron splice junctions (ISJ) reported for plants. The consensus sequence for the splice junctions is 9 bases at the 5'site and 7 bases at the 3' site (Figure 13.9.1). The additional bases are added at random to extend the length of the primers and provide potential polymorphic sites.

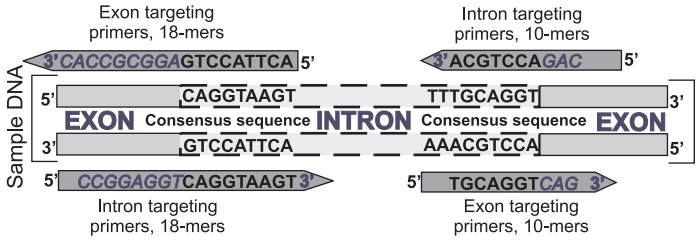


Figure 13.9.1. Principles of ISJ methodology

Primers were produced to generate products from the exon regions or from the intron regions. In total 12 primers were used at the different stages of studies (Annex 13.5). The primers were 10, 16 and 18 bases in length. Primers were noted using ISJ prefix. The polymorphic products were referred similarly to RAPD as follow:

- ISJ3-1 when referring to a product number counting from anode;

The dominant mode of inheritance of markers generated by all primers used in this study was verified in F_1 and F_2 samples derived from four intra- and interspecific *Lolium* crosses. The presence of a band was dominant over the lack of it.

Each PCR reaction was performed in a total volume 20 µl. Concentrations of reagents and thermal conditions are shown in Table 13.9.1. PCR products were loaded on 1.5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files. All bands that could be reliable read were scored either present (1) or absent (0).

Table 13.9.1. PCR conditions for ISJ markers

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	**1. Initial denaturation**	94°C	3
•**$MgCl_2$**	2.0 mM	**46 cycles**		
•**10 x Enhancer** containing betaine	2x	**2. Denaturation**	94°C	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 µM	**3. Annealing**	36°C or 48°C[1]	1
•**ISJ primer**	0.4 µM	**4. Elongation**	72°C	2.30
•**TFL polymerase** (Epicentre Technology)	1 U	**5. Final elongation**	72°C	5
•**DNA**	80 ng	**6. Soak**	4°C	∞

[1]36°C for primers of 10 bases in length, 48°C for primers of 16 and 18 bases in length

ANNEX 13.10. SIMPLE SEQUENCE REPEATS (SSR)

Simple Sequence Repeats (or SSR loci) arise due to local repetition of short sequence motifs. Each locus consists of di-, tri-, or tetranucleotides that are tandemly arrayed at particular chromosomal location. Polymorphism results from variation in repeat copy number. They are highly useful in genetic diversity and mapping studies. However, the high mutation rate and nature of this process causing that alleles identical in size are not always identical by descent seriously question the utility of the SSR approach in phylogeographic studies.

SSR were isolated from SSR-enriched *L. perenne* libraries by Jones et al. (2001). From ten primer pairs designed for SSR loci, seven were selected that were highly polymorphic and gave cross species amplification (Annex 13.5). Primers were noted following Jones et al. (2001) as A02, A07, A10, A11, C11, E10, H06. When multiple loci were detected, they were assigned using numerical order.

At the first step the PCR conditions were optimized to each pair of primers with respect to temperature and using the touch down phase. Each PCR reaction was performed in a total volume 20 µl. Concentrations of reagents and thermal conditions are shown in Table 13.10.1. PCR products were loaded on 2% (w/v) high resolution agarose gels (Sigma A4718)

containing 0.5 μg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files. All bands that could be reliable read were scored either present (1) or absent (0).

Table 13.10.1. PCR conditions for SSR markers

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	**1. Initial denaturation**	94°C	3
•**$MgCl_2$**	1.5 mM	**10 cycles (Touch down)**		
		2. Denaturation	94°C	1
•**10 x Enhancer** containing betaine	1x	**3. Annealing** •C11, E10 •A11, H06 •A02, A07, A10	50°C-1°C/cycle 60°C-1°C/cycle 65°C-1°C/cycle	1
		4. Elongation	72°C	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 μM	**20 cycles**		
		2. Denaturation	94°C	1
•**Forward primer** •**Reverse**	0.5 μM 0.5 μM	**3. Annealing** •C11, E10 •A11, H06 •A02, A07, A10	40°C 50°C 55°C	1
		4. Elongation	72°C	1
•**TFL polymerase** (Epicentre Technology)	0.75 U	**5. Final elongation**	72°C	5
•**DNA**	40 ng	**6. Soak**	4°C	∞

ANNEX 13.11. AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

The power of AFLP is based on genetic variation that exists between closely related species, varieties or cultivars. AFLP technology is a DNA fingerprinting technique that combines both classical restriction digestion and PCR-based fingerprinting. It is based on selective amplification of a subset of genomic restriction fragments using PCR. The steps of AFLP reaction are as follows (Figure 13.11.1):

- digestion of DNA with restriction endonucleases (Table 13.11.1),
- ligation of double-stranded DNA adapters to the ends of the DNA fragments to generate template DNA for amplification (Table 13.11.2),
- the sequence of the adapters and the adjacent restriction site serve as a primer binding sites for subsequent amplification of restriction fragments (Table 13.11.3),
- in the next PCR, selective nucleotides extending into the restriction fragments are added to the 3' ends of the PCR primers such that only a subset of the restriction fragments are recognized (Table 13.11.4),
- the subset of amplified fragments are analyzed by denaturing gel electrophoresis.

The AFLP procedure was based on Vos et al. (1995) with own modifications. The concentration of reagents in each step i.e., restriction digestion, ligation, pre-amplification and selective amplification as well as temperature regimes are given in Tables 13.11.1-13.11.4. After restriction digestion, ligation and pre-amplification, the efficiency of reactions was verified on 1% agarose gel containing 0.5 μg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files. Products of selective amplification were denatured with formamide at 94°C for 5 min. and separated on polyacrylamide gel at 45 W of constant power up to Xylene cyanol reached two third from the beginning. The gels were silver stained, scanned using a flatbed scanner and pictures were stored as .jpg files.

All bands that could be reliable read were scored either present (1) or absent (0). The dominant mode of inheritance of markers generated by all primers used in this study was verified in F_1 and F_2 samples derived from a cross between *L. multiflorum* cultivar Bartolini and *L. perenne* ecotype from New Zealand. The presence of a band was dominant over the lack of it. AFLP loci were named according to restriction sites flanked by adapters (*Mse*I, *EcoR*I) and three-base extensions of selective primers (e.g., McaaEact20). Numerical nomenclature was avoided in order to improve readability without necessity to look at the standard list for AFLP primer nomenclature.

Table 13.11.1. Restriction digestion

Reagent	Concentration	Thermal conditions
•**T4 ligase buffer** Epicentre Technology	1x	37°C/3 h 5 μl of the restriction digestion were checked on 1% agarose gel for 15 minutes.
•***Mse*I**	3 U	
•***EcoR*I**	3 U	
•**DNA**	180 ng	
Total volume	**18 μl**	

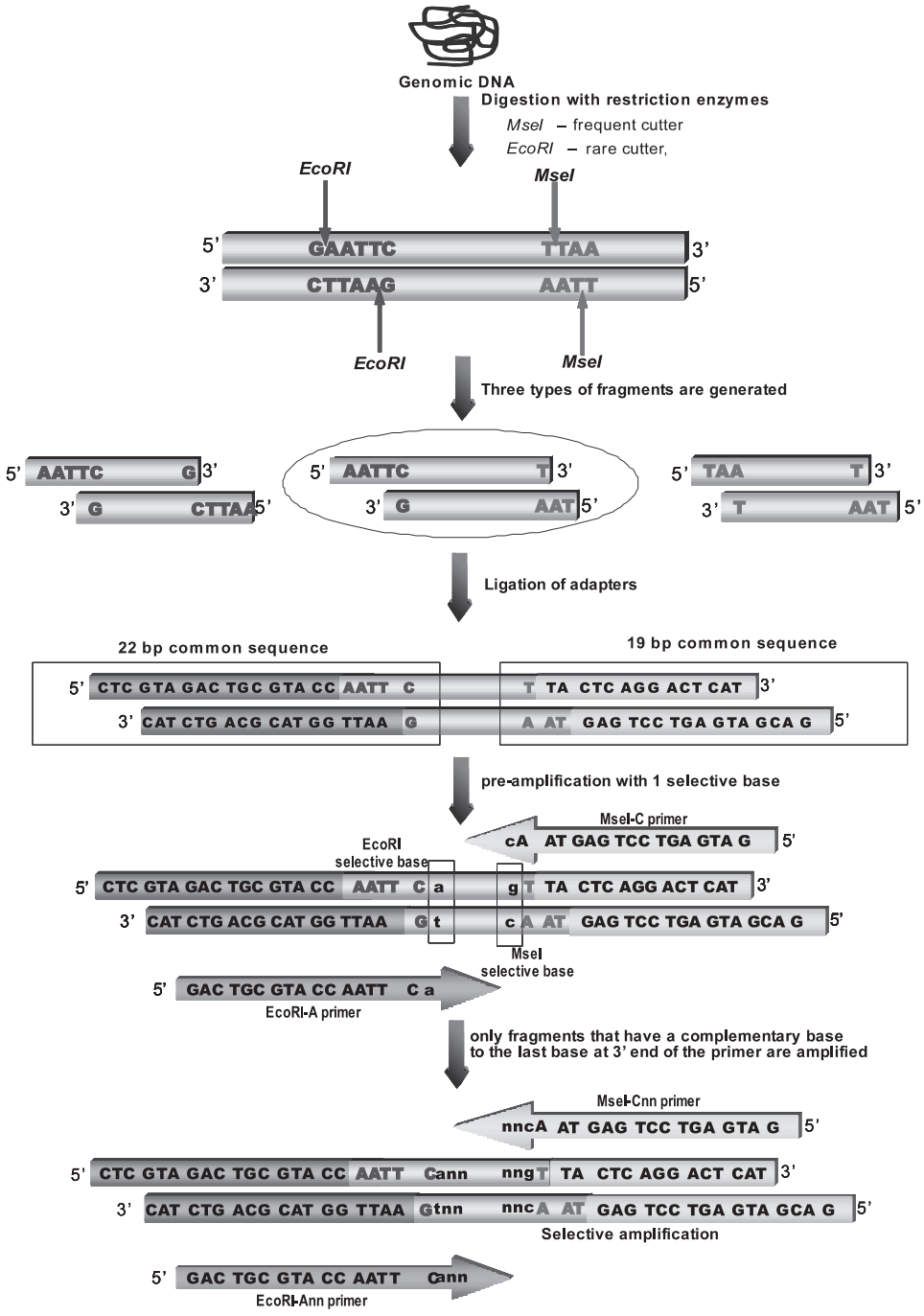


Figure 13.11.1. Principles of AFLP methodology

Table 13.11.2. Adapter ligation

Reagent	Concentration	Thermal conditions
•10 x T4 ligase buffer Epicentre Technology	1x	20°C/12 h 5 µl of ligation mixture were checked on 1% agarose gel for 15 minutes.
•ATP	0.4 mM	
•MseI-adapters	1.0 µM	
•EcoRI -adapters	0.1 µM	
•T4-DNA ligase Epicentre Technology	1 U	
•DNA	125 ng (12.5 µl from restriction digestion mixture)	
Total volume	**25 µl**	

Table 13.11.3. Pre-amplification

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•20 x PCR Buffer 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	1. Initial denaturation	95°C	1
•$MgCl_2$	1.5 mM	30 cycles (2-4)		
•10 x Enhancer containing betaine	1x	2. Denaturation	94°C	1
•dNTP dATP, dGTP, dCTP, dTTP	250 µM	3. Annealing	56°C	1
•MseC primer	0.25 µM	4. Elongation	72°C	1
•EcoA primer	0.25 µM	5. Final elongation	72°C	7
•TFL polymerase (Epicentre Technology)	0.5 U	6. Soak	4°C	∞
•DNA Ligation mixture diluted 1:9	1.5 µl,	5 µl of the pre-amplification mixture were checked on 1% agarose gel for 45 minutes.		
Total volume	**15 µl**			

Table 13.11.4. Selective amplification

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•20 x PCR Buffer 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	12 cycles (1-3)		
•$MgCl_2$	1.5 mM	1. Denaturation	94°C	0.5
•10 x Enhancer containing betaine	1x	2. Annealing with touch down phase	65°C -0.7°C per a cycle	0.5
•dNTP dATP, dGTP, dCTP, dTTP	250 µM	3. Elongation	72°C	1
•Mse selective primer	0.5 µM	28 cycles (4-6)		
•EcoRI selective primer	0.5 µM	4. Denaturation	94°C	0.5
•TFL polymerase Epicentre Technology	0.5 U	5. Annealing	56°C	0.5
•DNA Pre-amplification mixture diluted 1:49	2.5 µl	6. Elongation	72°C	1
•Total volume	10 µl	7. Soak	4°C	∞

ANNEX 13.12. SEQUENCE SPECIFIC AMPLIFICATION POLYMORPHISM (SSAP)

Sequence Specific Amplification Polymorphism (SSAP) is the retrotransposon-based molecular marker system that exploits the insertional polymorphism of retrotrasposon long terminal repeats (LTR) or terminal inverted repeats (TIR) in sequences of DNA transposons (Kumar et al. 1997). Transposons are present in all plant genomes and they show great variations in copy number and genome localization, even between closely related species. The methodology combines the general principle of AFLP with sequence-specific PCR to reveal sequence specific amplification polymorphism - SSAP (Figure 13.12.1). This approach is similar to AFLP in that genomic DNA is digested with restriction enzymes, enzyme-specific adapters are ligated to the restriction products and the resultant fragments are pre-amplified. The final step, selective amplification differs between two methods in that AFLP uses two different adapter-specific primers carrying selective bases to reduce the number of amplified products, whereas SSAP uses a single adapter-specific primer, together with a transposon-specific primer (Syed et aal. 2005). Thus SSAP requires one of AFLP primers in addition to a sequence specific primer generated on the basis of the terminal region of the mobile element. Sequences of transposon specific and AFLP primers are given in Annex 13.5.

The three specific primers were used to target three different transposon sequences. The first primer was produced from the terminal sequence of *L. perenne* DNA transposon, *Tpo1* (Langdon et al. 2003) belonging to the CACTA superfamily. The other two primers, preliminary assigned as LTR1 and LTR2 were designed to amplify *Ty1-copia* retrotransposon sequences. The RNAseH-LTR junction sequence from barley transposon, *BARE* was used in TBLASTN searches. Four promising sequences derived from *L. multiflorum* cDNA clone HL005F09-5 (Nº-AU248209 ver. AU248209.1), *Saccharum officinarum* cDNA clone SCSFLV1046E06 (Nº-CA300616 ver. CA300616.1), *Triticum aestivum* cDNA clone WHE4022-A08-A16 from meiotic anthers (Nº-CA500607 ver. CA500607.1) and *Oryza sativa* clone BR060005B10G04.ab1 similar to gag-*pol* protein (Nº-CA759285 ver. CA759185.1) were pre-aligned with CLUSTAL, then trimmed to roughly the same size, inverted and used in CLUSTAL multiple alignments. Two primers were derived:

- LTR1 based on *Lolium multiflorum* and *Oryza sativa* sequences,
- LTR2 based on *Saccharum officinarum* and *Triticum aestivum* sequences.

Because both primers produced bands on SSAP gel for *Lolium multiflorum* and *L. perenne*, showing that each transposon is present within them, the transposons revealed by LTR1 and LTR2 were further assigned as *Lolcopia1* and *Lolcopia2*.

All three primers were used in different combinations with AFLP primers thus yielding different insertion sites. The banding pattern related to the number and position of each transposon insertion. The dominant mode of inheritance of markers generated by all primers used in this study was verified in F_1 and F_2 samples derived from a cross between *L. multiflorum* cultivar Bartolini and *L. perenne* ecotype from New *Zea*land (BR3 x NZ15). The presence of a band was dominant over lack of the band.

The concentration of reagents in each step i.e., restriction digestion, ligation, pre-amplification and amplification as well as temperature regime are given in Tables 13.12.1-13.12.4. After restriction digestion, ligation and pre-amplification, the efficiency of reaction was veri-

fied on 1% agarose gel containing 0.5 μg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files. Products of selective amplification were denatured with formamide at 94°C for 7 min. and separated on denaturing polyacrylamide gel at 45 W of constant power up to Bromophenol Blue run out of the gel. The gels were silver stained, scanned using a flatbed scanner and pictures were stored as .jpg files. All bands that could be reliable read were scored either present (1) or absent (0). The bands were named by a transposon specific prefix and the product number from anode.

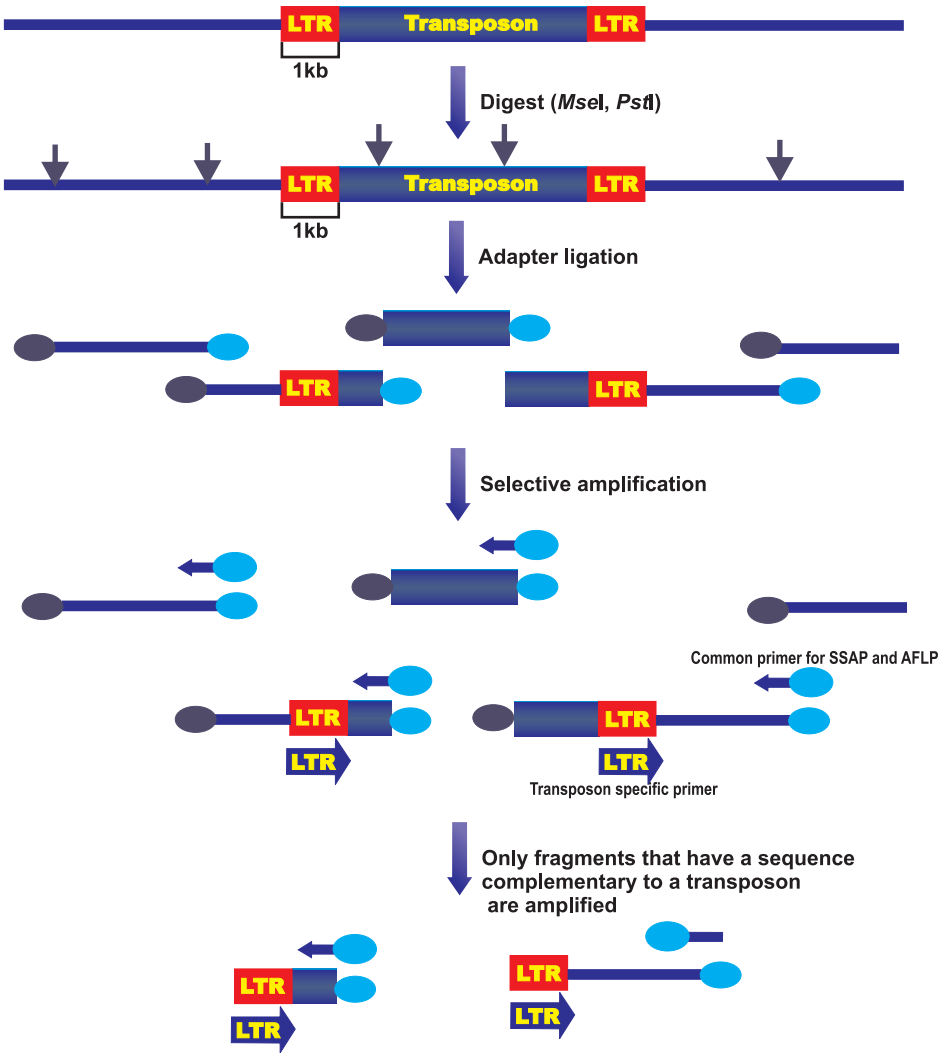


Figure 13.12.1. Principles of SSAP methodology

Table 13.12.1. Restriction digestion

Reagent	Concentration	Thermal conditions
•**T4 ligase buffer** (Epicentre Technology)	1x	37°C/12 h 5 µl of the restriction digestion were checked on 1% agarose gel for 15 minutes.
•***MseI*** (New England Biolabs)	3 U	
•***PstI*** (New England Biolabs)	3 U	
•**DNA**	360 ng	
Total volume	**18 µl**	

Table 13.12.2. Adapter ligation

Reagent	Concentration	Thermal conditions
•**10 x T4 ligase buffer** (Epicentre Technology)	1x	20°C/12 h 5 µl of ligation mixture were checked on 1% agarose gel for 15 minutes.
•**ATP**	0.4 mM	
•**MseI-adapters (MseAd)** (Annealed adapters)	1.0 µM	
•**PstI –adapters (PstAd)** (Annealed adapters)	0.5 µM	
•**T4-DNA ligase** (Epicentre Technology)	1 U	
•**DNA**	250 ng (12.5 µl from restriction digestion mixture)	
Total volume	**25 µl**	

Table 13.12.3. Pre-amplification

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	**1. Initial denaturation**	95°C	1
•**$MgCl_2$**	2.0 mM	**30 cycles (2-4)**		
•**10 x Enhancer** containing betaine	1x	**2. Denaturation**	94°C	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 µM	**3. Annealing**	60°C	1
•**Mse0 primer**	1.0 µM	**4. Elongation**	72°C	1
•**Pst0 primer**	1.0 µM	**5. Final elongation**	72°C	7
•**TFL polymerase** (Epicentre Technology)	0.6 U	**6. Soak**	4°C	∞
•**DNA**	10 ng 3 µl of ligation mixture,	5 µl of the pre-amplification mixture were checked on 1% agarose gel for 45 minutes.		
Total volume	**15 µl**			

Table 13.12.4. Selective amplification

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 mM Tris-HCl,	**13 cycles (1-3)**		
•**$MgCl_2$**	1.5 mM	**1. Denaturation**	94°C	1
•**10 x Enhancer** containing betaine	1x	**2. Annealing** with touch down phase	65°C -0.7°C per a cycle	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 µM	**3. Elongation**	72°C	1
•**Mse or Pst selective primer (from AFLP)**	0.5 µM	**22 cycles (4-6)**		
•**Transposon specific primer**	0.5 µM	**4. Denaturation**	94°C	1
•**TFL polymerase** Epicentre Technology	0.5 U	**5. Annealing**	56°C	1
•**DNA** from 1:4 diluted pre-amplification	2.5 µl	**6. Elongation**	72°C	1
•**Total volume**	**10 µl**	**7. Soak**	4°C	∞

ANNEX 13.13. BACTERIA SPECIFIC AMPLIFICATION POLYMORPHISM (B-SAP)

The system is based on the assumption that some plant genes including those encoding peroxidases of the class I have been derived from a common prokaryotic ancestor and they likely evolved through multiple duplications, conversions and translocations. The utility of primers derived from *KatG* gene encoding catalase- peroxidase and insertional element IS6110 of *M. tuberculosis* has been confirmed in phylogenetic studies of Poaceae, closely related *Pinus* taxa, liverworts from the genus *Pellia* and several others (Zielinski and Polok 2005). In the present work four types of bacteria-derived primers were used:

- 12 pairs of primers complementary to *KatG* gene of *M. tuberculosis*;
- a pair of outer primers complementary to IS6110 of *M. tuberculosis*;
- a pair of primers complementary to *rpo* gene of of *M. tuberculosis*;
- a pair of primers complementary to *pol* gene of of *M. tuberculosis*.

The PCR conditions were optimised in earlier studies (Zielinski and Polok 2005; Krzakowa et al. 2007). Each PCR reaction was performed in a total volume 20 µl. Concentrations of reagents and common thermal conditions are shown in Table 13.8.1. Number of cycles, annealing temperature and the time of elongation depended on pair of primers used. PCR products were loaded on 1.5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files.

All bands that could be reliable read were scored either present (1) or absent (0). The polymorphic products were referred as follow:

- - katG1-2 when referring to a band revealed by a set of katG primers n°-1 and the second product from anode
- - IS-1 when referring to a product number revealed by IS6110 primers counting from anode
- - rpo1 when referring to a product number revealed by *rpo* primers counting from anode
- - pol1 when referring to a product number revealed by *pol* primers counting from anode

The dominant mode of inheritance of markers generated by katG and IS6110 primers used in this study was verified in F_1 and F_2 samples derived from four intra- and interspecific *Lolium* crosses. The presence of a band was dominant over the lack of it.

Table 13.13.1. PCR conditions for B-SAP markers

Reagent	Concentration	Thermal conditions		
		Step	Temperature	Time [min]
•**20 x PCR Buffer** 200 mM $(NH_4)SO_4$, 1 M Tris-HCl, pH 9.0 at 25°C	1x 20 mM $(NH_4)SO_4$, 50 m M Tris-HCl,	**1. Initial Denaturation**	94°C	3
•**$MgCl_2$**	1.5 mM 2 mM (rpo, pol)	**N° of cycles** •katG2, katG5, katG6, katG10, katG11 - **35** •katG1, katG3, katG4, katG7, katG8, katG9, katG12 - **30** •IS6110, rpo, pol - **40**		
•**10 x Enhancer** containing betaine	1x	**2. Denaturation**	94°C	1
•**dNTP** (dATP, dGTP, dCTP, dTTP)	200 µM	**3. Annealing** •katG1, katG2, katG3, katG4, katG5, katG6, katG11, katG12 •katG7, IS6110 •katG8, katG9 •rpo, pol	 •53°C •52°C •50°C •51°C	 •1 •1 •1 •1
•**10 nucleotide primer**	1.0 µM	**4. Elongation** •katG1, katG2, katG3, katG4, katG5, katG6, katG7, katG10, katG11 katG12, IS6110, rpo •katG7, katG8, katG9, pol	72°C	•1.5 •1.0
•**TFL polymerase** (Epicentre Technology)	0.75 U 1 U (katG8, katG9, IS6110)	**5. Final elongation**	72°C	5
•**DNA**	100 ng	**6. Soak**	4°C	∞

ANNEX 13.14. SEQUENCE-TAGGED SITES (STS)

A number of markers generated by designing primers from a known sequence of the target genome were used in the present studies. Two types of sequences were employed, low copy genes and members of multigene families. The former were used for mapping studies whereas the latter both for mapping and phylogenetic studies. Primers were predominantly derived from *L. perenne* but also from *H. vulgare* and *A. sativa*. Moreover universal primers were used for amplification of internal transcribed region of rDNA (ITS1) and for amplification of a spacer between genes encoding leucine tRNA (Fiedorow et al. 2001).

At the first step the annealing temperature was optimized to receive a single band. After confirming the identity it was digested with enzymes previously selected on the basis of virtual digestion using NEB cutter. In cases of more than one product, this temperature was considered optimal at which reproducible pattern could be obtained. The PCR products were analysed with respect to amplification polymorphism or eluted from the gel, re-amplified and then subjected to restriction digestion. All bands that could be reliable read were scored either present (1) or absent (0). Sequences of all primers are given in Annex 13.5.

All PCR reactions were performed in a total volume 20 μl. Concentrations of reagents and cycling parameters depended on a sequence used and they are given in Table 13.14.1 and Table 13.14.2. PCR products either were subjected to restriction digestion or they were directly were loaded on 1.5%-2% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA; Sambrook et al. 1989) at 100 V constant power, visualized under UV light (312 nm), photographed and stored as .jpg files.

Table 13.14.1. Optimised concentration of reagents for amplification of STS markers

Name	Origin	$MgCl_2$	Enhancer	Primers	TFL polymerase	DNA
LOLPISO5A	*L. perenne*	1.5 mM	1 x	1.0 μM	0.75 U	100 ng
LOLPISO1A	*L. perenne*	1.5 mM	1 x	0.5 μM	0.75 U	100 ng
LOLLOIPB	*L. perenne*	2 mM	2 x	1.0 μM	0.75 U	100 ng
ASNL	*L. perenne*	2 mM	1 x	0.5 μM	0.75 U	80 ng
Trx	*L. perenne*	1.5 mM	1 x	0.5 μM	0.75 U	80 ng
Gln2	*L. perenne*	1.5 mM	1 x	0.5 μM	0.75 U	80 ng
LOLITS	*L. perenne*	1.5 mM	1 x	1.0 μM	0.75 U	100 ng
LTLHAB	*L. temulentum*	2 mM	1 x	1.0 μM	0.75 U	100 ng
ASN	*H. vulgare*	2 mM	1 x	0.5 μM	0.75 U	80 ng
AS1	*H. vulgare*	2 mM	1 x	0.5 μM	0.75 U	80 ng
HS1	*H. vulgare*	1.5 mM	1 x	0.5 μM	0.75 U	80 ng
BCD450	*H. vulgare*	2.5 mM	1 x	0.5 μM	0.75 U	80 ng
CDO504	*A. sativa*	1.5 mM	1 x	0.5 μM	0.75 U	80 ng
CDO1508	*A. sativa*	1.5 mM	1 x	0.5 μM	0.75 U	80 ng
ITS	universal	1.5 mM	1 x	0.5 μM	0.75 U	100 ng
t-RNA-leu	*Pellia sp.*	1.5 mM	1 x	0.5 μM	0.75 U	80 ng

*Concentrations of PCR buffer and dNTPs were the same i.e., 20 mM $(NH_4)SO_4$, 50 mM Tris-HCl, 200 μM dNTPs

Table 13.14.2. Optimised cycling paramethers

Name	Origin	N° of cycles	Annealing		Elongation
			Temperature	Time [min]	Time [min]
LOLPISO5A	*L. perenne*	35	62°C	1	1.5
LOLPISO1A	*L. perenne*	35	65°C	1	1.5
LOLLOIPB	*L. perenne*	40	62°C	1	1
ASNL	*L. perenne*	35	65°C	1	1
Trx	*L. perenne*	35	56°C	1	1
Gln2	*L. perenne*	35	56°C	1	1
LOLITS	*L. perenne*	35	59°C	1	1.5
LTLHAB	*L. temulentum*	40	60.5°C	1	1.5
ASN	*H. vulgare*	35	56°C	1	1
AS1	*H. vulgare*	35	53°C	1	1
HS1	*H. vulgare*	35	65°C	1	1
BCD450	*H. vulgare*	35	53°C	1	1
CDO504	*A. sativa*	35	65°C	1	1
CDO1508	*A. sativa*	35	65°C	1	1
ITS1	universal	35	52°C	1	1
t-RNA-leu	*Pellia sp.*	30	45°C	1	1

*Initial denaturation - 94°C for 3 min, denaturation - 94°C – 1 min, elongation - 72°C, final elongation - 72°C – 5 min.

ANNEX 13.15. POPULATION GENETIC STATISTICS

To study the extent of polymorphism and genetic diversity within *L. multiflorum* and *L. perenne* populations genetic statistics was adopted from Nei (1987).

Allele (gene) frequency — the frequency of a particular allele in a population

Codominant alleles (isoenzyme data)

$$p_i = P_{ii} + \frac{1}{2}\sum_{j \neq i} H_{ij} \qquad (Eq.\ 13.15.1)$$

p_i population frequency of the i^{th} allele at a locus
P_{ii} population frequency of ii homozygotes
H_{ij} population frequency of heterozygotes summarized over all j^{th} except for j=i

Dominant alleles (DNA data: RAPD, ISJ, SSAP)

$$q_i = \sqrt{Q_i} \qquad (Eq.\ 13.15.2)$$

$p_i = 1 - q_i$
p_i population frequency of dominant allele at i^{th} locus
q_i population frequency of recessive allele at i^{th} locus
Q_i population frequency of recessive homozygotes at i^{th} locus

Number of alleles per locus

$$A = \frac{\sum_{i=1}^{r} A_i}{r}$$

$$\overline{A} = \frac{\sum_{p=1}^{n} A}{n} \quad \text{and} \qquad (Eq.\ 13.15.3)$$

A the average number of alleles in a population over all loci
A_i the number of alleles at i^{th} locus in a population
$\overline{A}$ the mean number of alleles at i^{th} locus in a group of populations
r the number of loci
n the number of populations

Number of effective alleles per locus, n_e

$$n_e = \frac{1}{\sum p_i^2} \qquad (Eq.\ 13.15.4)$$

p_i population frequency of the i^{th} allele at a locus

Gene diversity (heterozygosity) in a population

Gene diversity (h) was introduced by Nei (1987) as a measure of genetic variation. It is defined unambiguously in terms of allele frequencies. The sample size effect on gene diversity is very low, since low frequency alleles hardly contribute to this quantity. Gene diversity can be used for any organism, whether it is haploid, diploid or polyploid and for any type of reproductive system. In practice, however, the word heterozygosity is widely used in the literature, so that both words can be used interchangeable. The average gene diversity (H) is calculated over all loci and indicates:

- the average proportion of heterozygotes per locus in a randomly mating population,
- the expected proportion of heterozygous loci in a randomly chosen individual,
- the probability that two randomly chosen genes from a population are different.

Gene diversity at a locus (heterozygosity) - h

The unbiased estimate of h recommended for randomly mating populations was calculated.

$$h = \frac{2n(1 - \sum_{i=1}^{m} p_i^2)}{2n - 1} \qquad (Eq.\ 13.15.5)$$

p_i population frequency of i^{th} allele at a locus
m the number of alleles
n the number of individuals

Gene identity at a locus - J

$$J = 1 - h \qquad (Eq.\ 13.15.6)$$

Average gene diversity at a locus over all populations, $\overline{h}$

$$\overline{h} = \frac{\sum_{i=1}^{n} h_i}{n} \qquad (Eq.\ 13.15.7)$$

h_i the gene diversity at i^{th} locus in a population
n the number of populations

Average (gene diversity) heterozygosity in a population, H

$$H = \frac{\sum_{i=1}^{r} h_i}{r} \qquad (Eq.\ 13.15.8)$$

h_i the gene diversity at i^{th} locus
r the number of loci

Gene diversities (heterozygosity) in populations divided into subpopulations

The method enables to divide the genomic variation into the interpopulational and intrapopulational variation.

Gene diversity in the total population (total gene diversity), H_T

$$H_T = 1 - J_T \qquad (Eq.\ 13.15.9)$$

$$H_T = 1 - \frac{\sum_k \sum_i p_{ki}^2 + \sum_{k \neq l} \sum_i p_{ki} p_{kl}}{s^2} \qquad (Eq.\ 13.15.10)$$

p_{ki} — the frequency of the i^{th} allele in the k^{th} subpopulation
p_{kl} — the frequency of the i^{th} allele in the l^{th} subpopulation
s — the number of subpopulations
$\sum_i p_{ki}^2 = J_k$ the gene identity in the k^{th} subpopulation

$\sum_i p_{ki} p_{li} = J_{kl}$ the gene identity between the k^{th} and l^{th} subpopulation

The average gene diversity within subpopulations, H_S

$$H_S = \frac{\sum_k H_k}{s} \qquad (Eq.\ 13.15.11)$$

H_k — the gene diversity in the k^{th} subpopulation
s — the number of subpopulations

The average gene diversity between subpopulations, D_{ST}

$$D_{ST} = H_T - H_S \qquad (Eq.\ 13.15.12)$$

H_T — the gene diversity in the total population
H_S — the average gene diversity within subpopulations

F statistics

The method enables to divide the genomic variation into the interpopulational and intrapopulational variation.

The coefficient of gene differentiation, G_{ST}

$$G_{ST} = \frac{D_{ST}}{H_T} \qquad (Eq.\ 13.15.13)$$

G_{ST} — the average gene diversity between subpopulations
H_T — the gene diversity in the total population

When there are two alleles at a locus, as in dominant DNA markers, this G_{ST} is equal to Wright's F_{ST} and it is relatively insensitive to assumptions about Hardy— Weinberg equilibrium, heterozygosity and levels of inbreeding.

The fixation indices, F_{IT}, F_{IS}

They are useful for understanding the breeding structure. They can be computed for the group of populations and for individual alleles and then averaged for a population

$$F_{IT} = 1 - \frac{H}{H_T} \qquad (Eq.\ 13.15.14)$$

F_{IT} — the deficiency or excess of average heterozygotes in a group of populations
H_T — the gene diversity in the total population

$$F_{IS} = 1 - \frac{H}{H_S} \qquad (Eq.\ 13.15.15)$$

F_{IS} — the deficiency or excess of average heterozygotes in each population
H_S — the average gene diversity

Nei's genetic identity and distance

Genetic distance is the extent of gene differences between populations or species. It can be measured as the number of nucleotide substitutions per locus. It can be estimated from polymorphism data. The principle of this method is that any difference in electrophoretic mobility (including lack of a band) is caused by at least one nucleotide difference at the DNA level and thus the average number of nucleotide differences per DNA fragment can be estimated from fragment frequency data. Isoenzymes detect only about 1/4 of amino acid substitutions in protein. Therefore to estimate the actual number of codon differences, the genetic distance must be multiplied by 4.

Unbiased standard genetic distance, D

$$D = -\ln I \qquad (Eq.\ 13.15.16)$$

I — normalized identity of genes, genetic identity

Normalized identity of genes, genetic identity, I

$$I = \frac{p_x p_y}{\sqrt{p_x^2 p_y^2}} \qquad (Eq.\ 13.15.17)$$

p_x — the frequency of i^{th} allele in a sample of 2n genes from population X
p_y — the frequency of i^{th} allele in a sample of 2n genes from population Y

Because the property that:

- I=1, two populations have identical allele (fragment) frequencies
- I=0, two populations share no alleles (fragments)

the I coefficient has been used for measuring the extent of genetic similarity between populations

Time of divergence, t

$$t = \frac{D}{2\alpha} \qquad (Eq.\ 13.15.18)$$

D — unbiased standard genetic distance
— the average rata of codon substitutions that is detectable by protein electrophoresis

14. ABBREVIATIONS

14.1. COMMON ABBREVIATIONS IN THE TEXT

Acc	— acetyl-CoA carboxylase gene
ACP	— Acid phosphatase, gene(s) — *Acp*
ACOH	— Aconitase hydratase, gene(s) — *Acoh*
ADH	— Alcohol dehydrogenase, gene(s) — *Adh*
AFLP	— Amplified Fragment Length Polymorphism
AS1	— *H. vulgare* asparagine synthetase gene
ASN	— *H. vulgare* L. asparaginase gene
ASNL	— *L. perenne* L-asparaginase gene
atpB	— chloroplast gene encoding the ß-subunit of ATP synthase
BEP clade	— Bambusoideae, Ehrhartoideae, Pooideae
BSC	— Biological Species Concept
B-SAP	— Bacteria Specific Amplification Polymorphism
CAP	— Cytosol aminopeptidase, gene(s) — *Cap*
cpDNA	— Chloroplast DNA
Core Pooids	— Aveneae, Bromeae, Poeae, Triticeae
EST	— Esterases, gene(s) — *Est*
gap3	— glyceraldehyde 3-phosphate dehydrogenase
Gln2	— *L. perenne* glutamine synthetase gene
GPI	— Glucose-6-phosphate isomerase, gene(s) — *gpi*
GS	— the glutamine synthetase gene family
GTDH	— Glutamate dehydogenase, gene(s) — *gtdh*
HK	— Hexokinase, gene(s) — *hex*
HS1	— *H. vulgare* gene encoding protein HS1
I	— Nei‘s genetic similarity (identity)
IDH	— Isocitrate dehydrogenase, gene(s) — *Idh*
IS6110 (IS)	— *Mycobacterium tuberculosis* insertion element
ISJ	— Intron Splice Junction
ITS	— Internal Transcribed Region
KatG	— *Mycobacterium tuberculosis* gene encoding catalase/peroxidase
katG	— marker system based on bacterial *katG* gene (B-SAP-katG)
LG	— Linkage group
LOD	— Likehood odds ratio statistics

Lolcopia1 — *Lolium Ty1-copia* retrotransposon
Lolcopia2 — *Lolium Ty1-copia* retrotransposon
LOLITS — ITS region of *L. perenne* ssp. *perenne*
LOLMTI — *L. multiflorum* mitochondrial gene
LOLPISO5A — *L. perenne* gene encoding pollen allergen Lol p I
LOLPISO1A — 3'end of *L. perenne* gene encoding pollen allergen Lol pI
LOLLOPIB — *L. perenne* gene encoding pollen allergen Lol p I b
LTHAB — *L. temulentum* nuclear gene encoding chlorophyll binding protein type II
LSD — Least Significant Difference
matK — chloroplast gene encoding maturase
MDH — Malate dehydrogenase NAD-dependent, gene(s) — *Mdh*
MDHP — Malate dehydrogenase NADP-dependent, gene(s) — *Mdhp*
MYA — Million years ago
multiflorum — *L. perenne* ssp. *multiflorum*
ndhF — chloroplast gene encoding a subunit of chloroplast NADH dehydrogenase
PCA — Principal Component Analysis
PER — Peroxidases, gene(s) — *Per*
perenne — *L. perenne* ssp. *perenne*
PGM — Phosphoglucomutase, gene(s) — *Pgm*
PGDH — Phosphogluconate dehydrogenase, gene(s) — *Pgdh*
PHY — the phytochrome gene family
pol — *M. tuberculosis* gene used in B-SAP
psbC — chloroplast gene encoding the photosystem II core polypeptide subunit P6 (psII 44 KD protein)
PVE — Phenotypic variance explained
QTL — Quantitative Trait Loci
rbcL — chloroplast gene encoding the large subunit of 1,5-bisphosphate carboxylase/oxygenase
rpoC2 — chloroplast gene encoding ß-subunit of RNA polymerase
rpo — *M. tuberculosis* gene used in B-SAP
rps4 — chloroplast gene encoding the S4 subunit of plastid ribosome
RAPD — Random Amplified Polymorphic DNA
rDNA — nuclear ribosomal DNA
5.8S rDNA — nuclear gene encoding 5.8S rRNA
18S rDNA — nuclear gene encoding 18S rRNA (small subunit)
26S rDNA — nuclear gene encoding 26S rRNA (large subunit)
RFLP — Restriction Fragment Length Polymorphism
RUBISCO — 1,5-bisphosphate carboxylase/oxygenase
S, Z — self-incompatibility loci
SKDK — Shikimate dehydrogenase, gene(s) — *Skdh*
SOD — Superoxide dismutase, gene(s) — *Sod*
SRF — Seedling Root Fluorescence

SSAP	— Sequence Specific Amplification Polymorphism
SSR	— Simple Sequence Repeats
STS	— Sequence Tagged Sites
tb1	— *teosinte branched1* — maize locus responsible for transition from teosinte to maize
Tcb1-s, *tcb1*	— maize locus responsible for hybridization with teosinte
Tpo1	— *Lolium* DNA transposon from CACTA family
tRNA-leu	— spacer between genes encoding leucine tRNA
trnF	— chloroplast gene encoding tRNA-Phe (GAA)
trnL	— chloroplast intron ranging from 350-600 bp
trnS	— chloroplast gene encoding tRNA-Ser (UGA)
trnL-F	— the intergenic spacer between the *trnL* exon and the *trnF* gene
Trx	— *L. perenne* thioredoxin gene
waxy	— granule bound starch synthase gene
UPGMA	— Unweighted Pair Group Method with arithmetic Averages

14.2. SPECIES LATIN NAMES AND COMMON NAMES

Aconitum columbianum		4.4.3
Aconitum noveboracense		4.4.3
Aegilops cylindrical		*9*.3
Agave deserti		4.4.3
Agrostis capillaries		5.4
Agrostis palustris		5.4
Aneura pinquis		3.5; 8.1
Avena fatua		9.3
Avena sativa		4.4.4; 6.2.2; 6.3.1; 9.2; 9.3
Avena strigosa	bristle oat	1.5; 9.2; 9.3
Avena wiestii		9.3
Arabidopsis thaliana	arabidopsis	4.4.3, 7.4.2; 9.2; 9.3
Arabidopsis lyrata		4.4.3
Arrhenatherum elatius		9.3
Bromus tectorum		4.4.1
Buchloe dactyloides		4.4.2
Calamagrostis arundinacea		6.4.3
Conocephalus conicum		8.1
Conocephalum salebrosum		8.1
Dactylis glomerata		9.3
Delphinium variegatum		4.4.3
Drosophila melanogaster	fruit fly	4.4.4

Drosophila willistoni		4.4.3
Festuca arundinacea	tall fescue	1.3; 1.5; 3.5; 9.3
Festuca fontgueri		1.4
Festuca gigantea		1.4; 1.5; 3.5
Festuca pratensis	meadow fescue	1.3; 1.4; 1.5; 3.5; 4.4.1; 8.2; 8.3; 8.4; 9.2; 9.3
Festuca rubra		9.3
Glycine max		4.4.4.
Glicine soja	soybean	4.4.4; 5.4
Helianthus anomalus		6.1
Helianthus annuus		6.1
Helianthus petiolaris		6.1
Hordeum bulbosum		1.5
Hordeum marinum		9.3
Hordeum spontaneum	wild barley	4.4.1.; 4.4.4
Hordeum vulgare	barley	1.5; 3.5; 4.1; 4.2; 6.2.2; 6.3.1; 7.1; 9.2; 9.3
Iris fulva		6.1
Iris brevicaulis		6.1
Lolium canariense		1.1; 8.2
Lolium x *hybridum*		1.2; 1.5; 3.5; 4.1; 4.2; 4.3.1; 4.3.3
Lolium loliaceum		1.1; 1.2; 1.4; 5.1; 5.2; 5.3; 5.4; 8; 9.2; 9.3
Lolium multiflorum	italian ryegrass, annual ryegrass	1-7
Lolium multiflorum var. westerworldicum	Westerwold ryegrass	1.2
Lolium perenne	perennial ryegrass	1-7
Lolium perenne ssp. m*ultiflorum*		8-10
Lolium perenne ssp. *perenne*		8-10
Lolium persicum	persian darnel	1.1; 1.2; 3.5; 5.1; 5.2; 5.3; 5.4; 8; 9.2; 9.3
Lolium remotum		1.1; 1.2; 1.4; 1.5; 3.5; 4.1; 5.1; 5.2; 5.3; 5.4; 8; 9.2; 9.3
Lolium rigidum		1.1; 1.2; 1.4; 1.6; 4.1; 4.4.1; 5.1; 5.2; 5.3; 5.4; 8; 9.2; 9.3

Lolium rigidum var. *rottbollioides*		1.4
Lolium subulatum		1.4
Lolium temulentum	darnel	1.1; 1.2; 1.4; 1.5; 1.6; 3.5; 4.1; 5.1; 5.2; 5.3; 5.4; 6.2.2; 6.3.1; 8; 9.2; 9.3
Lycopersicon esculentum	tomato	6.1; 7.1
Medicago truncatula		9.3; 10.5
Mimulus cardinalis		7.1
Mimulus guttatus	monkeyflowers	6.1; 7.1
Mimulus lewisii		7.1
Mimulus nasutus	monkeyflowers	6.1; 7.1
Mycobacterium tuberculosis		1.6; 6.2.2; 6.3.1; 6.3.3; 6.3.4; 6.4.3; 7.2; 7.3; 9.2; 9.3; 10.6
Oryza longistaminata		7.4.2
Oryza sativa	rice	4.2; 4.4.4; 5.4; 7.4
Oryza sativa ssp. *japonica*		7.4.1
Oryza sativa ssp. *indica*		7.4.1
Oxalis tuberosa	oca	5.4
Pellia endiviifolia		1.6; 3.5
Pennisetum glaucum	pear millet	6.1
Penisetum violaceum		6.1
Pinus cembra	stone pine	4.4.1; 4.4.3
Pinus mugo	dwarf pine	1.6; 4.1; 4.4.3
Pinus pumila		4.4.3
Pinus sylvestris	Scots pine	1.6; 3.5; 4.1; 4.4.1; 4.4.3
Pinus uliginosa	peat-bog pine	4.4.3
Pisum abyssinicum		4.4.4
Pisum elatius		4.4.4
Pisum fulvum		4.4.4
Pisum humile		4.4.4
Pisum sativum	pea	4.1; 4.4.1; 4.4.4; 6.4.3; 7.1; 9.3; 10.5
Pleurozium schreberi		10.4
Polygonatum verticillatum		7.4
Puccinia coronata f. sp. *lolii*		7.2.1
Poa trivialis		1.3; 9.3
Poa pratensis		8.2; 8.3; 8.4; 9.2; 9.3
Pseudoroegneria spikata		4.4.1
Raphanus sativus	cultivated radish	8.4.3
Raphanus raphanistrum	jointed charlock	8.4.3
Quercus petraea		7.1; 7.4.2
Quercus robur		7.1; 7.4.2

Saccharomyces cerevisiae	yeast	4.4.4
Saccharum officinarum		4.2
Secale cereal	rye	4.4.4.; 6.4.1; 9.2; 9.3
Senecio gallicus		4.4.1
Setaria italica	foxtail millet	7.1
Setaria viridis	green millet	7.1
Solanum lycopersicoides	wild nightshade	6.1
Solidago virga aurea		7.4
Solidago virga aurea ssp. *virga aurea*		7.4
Solidago virga aurea ssp. *alpestris*		7.4
Triticale		9.2; 9.3
Triticum aestivum	wheat	4.2; 4.4.4; 5.4; 9.2; 9.3
Triticum dicoccoides	emmer wheat	4.4.1; 7.1; 7.4.2
Triticum durum		7.1
Triticum spelta	spelt	5.4
Zea mays	maize	4.4.3; 7.1
Zea mays ssp. *mexicana*	teosine	4.4.3
Zea mays ssp. *parviglumis*	teosinte	4.4.3; 7.1
Zizania latifolia		4.4.4.

15. SUMMARY

The genus *Lolium* is classified within the family Poaceae, subfamily Pooideae, tribe Poeae. The genus consists from about eight species that are native to Europe, temperate Asia and North Africa although most species have been widely distributed around the temperate areas of the world. All species are diploid, mostly annual grasses, with the only exception of *L. perenne* that is the only perennial form within the genus. Four of them, *L. loliaceum*, *L. persicum*, *L. remotum* and *L. temulentum* are autogamous species whereas *L. multiflorum*, *L. perenne* and *L. rigidum* are allogamous species. *L. canariense* is supposed to be intermediate with respect to the mode of reproduction. Among them, *L. multiflorum* and *L. perenne* are important fodder and turf grasses. The species classification within the genus *Lolium* is based on morphological characters but, owing to overlapping ranges of variation, it has been a subject of a continuous debate for many years. The greatest controversy is associated with two allogamous species. *L. multiflorum* and *L. perenne*, although the taxonomic position of the others is also unclear. Notwithstanding the fact of crossability and full fertility of F_1 and next generation hybrids, *L. multiflorum* and *L. perenne* are consequently classified as two separate taxa. On the other hand, our earlier results suggest that there are no reproductive barriers between them and thereby, they can not be regarded as biological species under the BSC concept. Apart from *L. multiflorum* and *L. perenne*, there is no agreement on the position of *L. loliaceum* as well as the discussion has been going on about a common ancestor of the genus. With the power of the most advanced molecular technologies these controversies are likely to be solved. Unfortunately, in spite of intensive breeding activities and advanced research aimed at improving the turf quality, very little attention has been paid to the evolutionary relationships within the genus.

Therefore, the aim of this research was to review the evolution within the genus *Lolium* using the broad spectrum of biometrical methods and high throughput molecular technologies. For the first time, the population genetic and phylogenetic methodologies were combined with genetic mapping in order to elucidate the relationships between two botanical species *L. multiflorum* and *L. perenne* and determine whether they can be regarded as distinct biological species. The presence of a reproductive barrier was studied at the level of a genetic map and QTLs potentially involved in the species differentiation were searched for. The attempts to clarify the evolutionary relationships within the genus were also undertaken. Besides commonly known technologies, own marker systems were offered. Additionally, the other members of “Core Pooids” i.e., Poeae, Aveneae and Triticeae were compared with seven *Lolium* species in order to estimate the level of similarity between *Lolium* and the other representatives of grasses.

At different stages of analyses, in total 45 populations representing ecotypes and cultivars of *L. multiflorum* and *L. perenne*, two F_2 populations derived from interspecific crosses between *L. multiflorum* and *L. perenne* (470 individuals), one *L. multiflorum* F_2 population (205 individuals) and one *L. perenne* F_2 population (44 individuals) were used. Moreover, the remaining representatives of the genus *Lolium* i.e., *L. loliaceum*, *L. persicum*, *L. remotum*, *L. temulentum*, *L. rigidum* were used in phylogenetic studies in addition to closely related *F. pratensis*, *P. pratensis* and representatives of the Triticeae tribe (*H. vulgare*, *T. aestivum*, *S. cereale*, *Triticale*) and Aveneae tribe (*A. sativa* and *A. strigosa*). The model species *A. thaliana* was used as outgroup.

A wide range of morphological, statistical and molecular methods was used to asses genetic variation, construct genetic map, map QTLs and resolve phylogenetic relationships within the genus. In total 23 quantitative, morphological traits including seedling characters, vegetative and generative traits were analysed in addition to seedling root fluorescence. Three types of random, genome scanning markers, RAPD, ISJ and AFLP were employed at different stages of studies. Insertional polymorphism was analysed using SSAP. Furthermore SSR markers and STS markers derived from *Lolium* and cereal sequences coupled with restriction site polymorphism analysis were used in genetic mapping and phylogenetic studies. Own marker system was elaborated based on primers derived from bacterial sequences and used in mapping and phylogenetic studies (B-SAP). The analysis of organelle DNA (cpDNA, mtDNA) was employed in genetic diversity and phylogenetic studies whereas isozymes were used in genetic diversity analyses and genetic mapping. In total 67 RAPD primers, 12 ISJ primers, 36 AFLP primer combinations, nine SSAP primer combinations specific to the DNA transposon *Tpo1*, and two *Ty1-copia* retrotransposons (*Lolcopia1*, *Lolcopia2*), seven SSR pair of primers, 12 STS sets of primers derived from low copy genes or RFLP probes originated from *L. perenne*, *L. temulentum, H. vulgare* and *A. sativa*, a pair of universal primers amplifying ITS1 region, a pair of primers specific to *L. perenne* ITS region, a pair of primers amplifying spacer between tRNA-leu genes and 15 sets of B-SAP primers complementary to *M. tuberculosis* sequences were used. Among B-SAP primers, apart from 12 sets derived from *KatG* gene encoding catalase-peroxidase, primers complementary to insertion element IS6110, and hot regions of *rpo* and *pol* genes were used. During studies a wide range of statistical methods and programmes from the field of population genetics, phylogenetics, map construction and QTL mapping were employed.

The morphological analysis of taxonomic traits did not confirm the separation of *L. multiflorum* and *L. perenne* into two species. They were similar in the majority of studied characters with the overlapping range of variation. Both species had the same genetic structure as indicated by a wealth of molecular markers including enzymes, chloroplast and mitochondrial DNA, RAPD, ISJ. All markers analysed revealed nearly the same level of polymorphism in *L. multiflorum* and *L. perenne* and the markers specific to a species were hardly found. High molecular similarity strongly supported the classification of *L. multiflorum* and *L. perenne* as a single species. To confirm these results, transposon-based markers, highly effective in evolutionary studies were used for the first time. Three different transposon sequences were applied including, DNA transposon from the CACTA family, *Tpo1* and two *Ty1-copia* retrotransposons, *Lolcopia1* and *Lolcopia2*. The analy-

ses of insertional polymorphism fully confirmed the previous data that *L. multiflorum* and *L. perenne* had the common gene pool and they can not be regarded as biological species. However, the higher activity of transposons in *L. multiflorum* suggests further diversification of both species. The association of distorted regions on genetic map of *L. multiflorum* and *L. perenne* with transposons was another confirmation of their role in the early steps of evolution of the genus *Lolium*.

Comparison of marker distortions in intra- and interspecific F_2 populations derived from four different crosses and genetic mapping of 502 DNA, enzymatic markers and seedling root fluorescence provided more evidences that a relatively little genetic incompatibilities were between *L. multiflorum* and *L. perenne* and no signs of a reproductive barrier were observed. The majority of markers segregated in the Mendelian fashion. The level of distortions was comparable in intra- and interspecific crosses. Selection against transposon insertion provided evidences that intraspecific mechanisms protecting from undesired mobilisation of transposons are well working in the populations derived from crosses between *L. multiflorum* and *L. perenne*. Moreover, seedling root fluorescence that is regarded as marker discriminating *L. multiflorum* from *L. perenne* was found to be encoded by two complementary genes located on LG1. In intra- and interspecific populations either both genes or one of them can segregate. This model of inheritance explains well the difficulties in species separation and stabilisation of the seedling root fluorescence level in cultivars of these species frequently encountered by breeders.

There are no differences with respect to the occurrence, magnitude and mechanisms underlying heterosis in inter- and intraspecific crosses. Heterosis resulted from dominance and gene interactions and it was not correlated with genetic distance between parents. In total 145 QTLs were responsible for *L. multiflorum* and *L. perenne* evolution however, the majority of them were related with the process of domestication. According to historical sources *L. multiflorum* is a domesticated form of *L. perenne* that was selected in the Middle-Ages, and after that elevated to a species status. The domestication process is controlled by many major QTLs in addition to several minor QTLs that are located within nine domestication syndrome regions on six linkage groups. All these data fully confirmed our previous view that *L. multiflorum* and *L. perenne* can not be regarded as biological species. Therefore their taxonomic rank should be lowered and the subspecies level seems to be more appropriate. Implementing the same philosophy as it is done for maize and teosinte, or indica and japonica forms in rice, it was proposed to classify *L. multiflorum* and *L. perenne* with a rank of subspecies i.e., *L. perenne* ssp. *perenne* for a more primitive form and *L. perenne* ssp. *multiflorum* as a domesticated form in agreement with the Integrated Taxonomic System of the USA.

Once the evolutionary relationships between *L. multiflorum* and *L. perenne* were resolved, it was possible to clarify the phylogeny of the whole genus *Lolium*. At this point plenty molecular methods were used to estimate the level of divergence between seven species of the genus *Lolium*, *L. loliaceum*, *L. persicum*, *L. remotum*, *L. temulentum*, *L. multiflorum*, *L. perenne* and *L. rigidum*. Moreover, closely related species, *F. pratensis* and *P. pratensis* were used to help the tree rooting. No studies can be compared with the present in the terms of number of methods and markers used to reliably establish the evolutionary relationships within the genus *Lolium*. The restriction analysis of the

L. multiflorum mitochondrial gene, LOLMTI, *L. perenne* pollen allergen gene, LOLPISO5A, analysis of cpDNA and mtDNA in addition to 2894 nuclear molecular markers including RAPD, ISJ, SSR, AFLP, SSAP based on *Tpo1*, *Lolcopia1* and *Lolcopia2* transposons, and STS derived from *Lolium* species, cereals and *M. tuberculosis* enabled to resolve phylogenetic relationships within the genus *Lolium*. The emerging view from these studies was that the most probable common ancestor of the genus *Lolium* had the closest affinity to the subgenus of *Festuca*, *Schedonorus*, of which *F. pratensis* is a representative. The genus can be divided into two clades representing two different lineages that evolved independently from the common ancestor. The first clade (Temulentum) consists of *L. persicum*, *L. remotum* and *L. temulentum* and diverged about 2.7 MYA, while the second (Perenne) consists of *L. loliaceum*, *L. perenne* ssp. *multiflorum*, *L. perenne* ssp. *perenne* and *L. rigidum* and diverged about 2.35 MYA. Self-fertility arose independently in both clades. Within Temulentum clade, *L. persicum* diversified the first about 1.3 MYA and the separation between *L. remotum* and *L. temulentum* can be dated to 0.95 MYA. Within Perenne clade, *L. rigidum* split into a separate species about 1.4 MYA whereas *L. loliaceum* about 1.2 MYA. The latter originated through several point mutations responsible for self-pollination. The diversification of *L. perenne* populations started 167 000 years ago. As a response for more harsh conditions during glaciations the perenniality came into existence in more northern populations. At the same time more southern populations adapted to milder climate in south parts of Europe. However, because in the Quaternary Ice Ages *L. perenne* was widespread and extinct alternately, its populations survived in refugia in south and central part of Europe, and thus, there was no sufficient isolation between them and gene flow disabled the birth of reproduction barriers. Only during the Middle-Ages, unconscious selections led to the development of lines similar to the present *L. multiflorum*. Their elevation to a species rank resulted in the creation of *L. multiflorum* that can be regarded as the only domesticated grass. The low similarity between the genus *Lolium* and cereals can cause difficulties in using anchor probes, EST or STS from cereals.

In the course of the present studies several new approaches were used including the application of transposon-based polymorphism to genetic diversity and phylogenetic analyses. Furthermore, for the first time in *Lolium* the genetic map was used to address evolutionary problems and to map QTLs related with domestication process. The map was also unique because it consisted from a wide range of different marker categories that prevented from marker clustering and forming the gaps. For the first time transposon sequences were mapped. Moreover, it was possible to map 18 enzymatic loci, prove that seedling root fluorescence is encoded by two complementary genes and map as many as 145 QTLs, responsible for taxonomic characters. The current studies together with historical resources for the first time confirmed that *L. multiflorum* and *L. perenne* are not biological species, and that the differences between these two forms are related with the domestication process of *L. multiflorum*. Consequently, the QTLs responsible for the domestication were mapped. In spite of clarifying the status of *L. multiflorum* and *L. perenne*, it was possible to prove that the common ancestor of the genus has the closest affinity to *Schedonorus* and two clades, named here Temulentum and Perenne evolved independently from it. These clades are in general agreement with the mode of reproduction except for autogamous *L. loliaceum* belonging to Perenne clade.

A by-product of all these analyses was the elaboration of a new marker system based on different bacterial genes, but mainly on catalase-peroxidase from *M. tuberculosis*. The system proved to be highly effective in phylogenetic analyses and revealing species specific markers. The resolution of the tree based on katG markers was comparable to that based on multi-marker approach and therefore the system can be used an alternative to using many different marker types. Genetic mapping studies enabled to locate markers derived from bacteria on *Lolium* linkage map. The markers had the dominant mode of inheritance i.e., the presence of a band is dominant over its lack. Moreover, these types of markers rarely show segregation distortions. The linkage between katG markers and peroxidase loci support the idea that katG primers complementary to the *M. tuberculosis* catalase-peroxidase gene amplify plant peroxidases. The linkage between katG and the other enzymatic loci proves that these markers are predominantly correlated with genes encoding enzymes. It was proposed to name this method as **Bacteria Specific Amplification Polymorphism (B-SAP)**. And finally, all these analysis were coupled with estimation of marker efficiency with respect to the costs and utility in different areas of genetic evolutionary research.

REVIEWERS' COMMENTS

With a great interest I have read the Kornelia Polok's book, in which she presented results of her long-standing research on evolution of grasses from the genus *Lolium*. A central point of reference to the author is the Mayr's Biological Species Concept (BSC) under which she verifies analysed taxa. Combining excellently the data from the field of population genetics, genome mapping and molecular phylogeny she draws the comprehensive picture depicting the origin of studied species and relationships between them in a new light. The book describes the dynamics of evolutionary changes at the molecular level with special emphasis on transposons as the driving forces behind the evolutionary process. Similarly, the role of quantitative trait loci (QTLs) in the evolution and domestication of the species from the genus *Lolium* is well illustrated by a number of domestication syndrome regions identified. I was very impressed by a grand scale of research, a wealth of methods applied and a very well design of the monograph. The author is exceptionally clear in discussing of population processes, species diversification, different consequences of gene flow and introgression. The writing style is simple and direct, the content fabulous and the perspective illuminating.

Prof. dr hab. Roman Zielinski

While reading a monograph, it seems incredible that this is the work of one person, the more drawing up based on own results. The author unequivocally concludes that *Lolium multiflorum* and *L. perenne* should be regarded as one biological species (nowadays they are classified as separate botanical species). However, the number of incontrovertible evidences is sine qua non to dare to revise the current classification. Such apparent evidences are provided by Kornelia Polok in the form of results emerging from numerous experiments. The detailed description of methodology makes the book, apart from a mine of knowledge about studied species, a very valuable reference source for further inquiry, especially about setting up and conducting experiments related with quantitative traits' analyses, biotechnology (in particular, application of molecular markers to assessing the level of polymorphism and constructing genetic maps) as well as phylogenetic studies. Unconventional organizing the monograph into individual chapters, yet fitting together into a logical whole, makes the work remarkably clear. With both extraordinary lightness and preciseness, the author was able to pass the reader on results of her explorations. And the splendid discussion carried out against a background of extensive scientific literature! The writing style is easy-reading and concise, indeed elegant. This is a valuable book with a vital and major impact on knowledge about evolution of the genus *Lolium*.

Prof. dr hab. Tadeusz Adamski

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Kornelia Polok, PhD in biology, genetics, biological studies at the Silesian University, Katowice, Poland, master thesis on somaclonal variation in *Arabidopis thaliana*. In her PhD thesis, using doubled haploids, she clarified the genetic basis of heterosis in *Hordeum vulgare*. In 1996 she moved to the Department of Genetics, University of Szczecin, where she was involved in population genetic and evolutionary studies of several plant taxa but also molluscs (*Dreissena*) and bacteria (*Mycobacterium tuberculosis*). After two years, in 1998, she moved to the University of Warmia and Mazury in Olsztyn, where, together with Prof. dr hab. Roman Zielinski, she organized the Department of Genetics at the newly established Faculty of Biology.

In 1997 she spent five months in the Plant Molecular Biology Laboratory, Institute of Bioorganic Chemistry, Poznań, Poland. In 2005 and 2006 she was visiting the University of Dundee (Andrew Flavell laboratory, UK), where she developed transposon technology in *Lolium* species. She was the first to apply transposon based analyses to clarify phylogenetic relationships between closely related *Pinus sylvestris*, *P. mugo*, *P. uliginosa* and *P. rhaetica*. She also co-operates with researchers from the University of Hannover (Germany) on transgenic pea, Wageningen University (Netherlands) on isolation of pea genes using the *Medicago truncatula* library and the University of Southampton (UK) on genetic improvement of underutilised crops. Her contacts with the Tatra National Park aim at developing molecular methods useful in the biodiversity protection.

She has been involved in European projects as well as she has an experience in the project evaluation within EU Framework Programmes. She is active in graduate and postgraduate education, mainly delivering lectures from molecular genetics, plant genomics, genome annotation, molecular evolution, mutagenesis and quantitative genetics. She has also been a supervisor of several MSc candidates from biology and biotechnology at the University of Warmia and Mazury in Olsztyn.

In her research work, Kornelia combines knowledge from applied and evolutionary genetics. Topics range from molecular evolution and population genetics of several plant taxa (e.g., Poaceae, *Rosa* sp., liverworts from the genus *Pellia* and *Aneura*, closely related *Pinus* species), genetic basis of quantitative inheritance including QTL mapping, role of QTLs in plant evolution as well as genetic basis of heterosis, genetic improvement of underutilised species including mapping and cloning genes responsible for important characters (Al tolerance in *Avena strigosa*, lodging resistance and dwarfism in *Pisum sativum*).

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