

Genetic variability of Scots pine (*Pinus sylvestris* L.) in maternal regions of provenance

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ABSTRACT

The analysis of chosen selected Scots Pine (*Pinus sylvestris* L.), populations representing different seed regions: 107 (Międzyzdroje), 305 (Woziwoda), 206 (Strzałowo), 208 (Białowieża), 504 (Bolesławiec), 606 (Józefów) were performed using 10 isoenzyme markers: *Gdh* (E.C.1.4.1.2), *Sdh-A*, *Sdh-B* (E.C.1.1.1.25), *Pgd-B* (E.C.1.1.1.44), *Mdh-A*, *Mdh-C* (E.C.1.1.1.37), *Got-A*, *Got-B*, *Got-C* (E.C.2.6.1.1), *Dia-C* (E.C.1.8.1.4). There were calculated following genetic parameters: allelic frequencies, observed and expected heterozygosities, and Wright's fixation indexes. In populations, the results of analysis indicated presence of rare alleles. In all study populations, the average effective number of alleles per locus was 1.46 and was lower than expected number of alleles per locus of 1.93. The results for the effective number of alleles of the population per locus were as follows: Strzałowo and Białowieża 1.54, Bolesławiec 1.48, Józefów 1.44, Świnoujście 1.42, Woziwoda 1.35. Average observed heterozygosity in the studied populations was calculated at the level of 0.26 and it was lower than the expected heterozygosity at 0.28. For populations, the level of heterozygosities were as follow: Strzałowo 0.35, Bolesławiec and Białowieża 0.25, Józefów 0.27, Świnoujście 0.23, Woziwoda 0.26. In particular loci level of heterozygosity was different, as the most heterozygous *Mdh-C* locus was estimated, while minimum *Got-C*.

Significant differences in allele frequency of Hardy-Weinberg deviation equilibrium were found in 10 cases: Strzałowo (*Got-C*), Bolesławiec (*Got-B*), Białowieża (*Got-B*, *Gdh*), Józefów (*Mdh-C*), Międzyzdroje (*Got-B*, *Got-C*, *Mdh-C*), Woziwoda (*Got-C*, *Gdh*). All studied populations had a lower effective number of alleles per locus (N_e) comparing to the observed number of alleles (N_a). Wright's fixation indices were negative for populations: Strzałowo (-0.09), Józefów (-0.03), Międzyzdroje (-0.01) and positive for: Białowieża (0.12), Woziwoda and Bolesławiec (0.07). The extremely high Wright's fixation index (0.12) was observed for Białowieża population.

KEY WORDS

genetic diversity, molecular markers, gene pool conservation

INTRODUCTION

The Scots pine is particularly widespread species. It is present on both European and Asian continents, and it has the greatest natural range among *Pinus* species.

Most of the data used as basis in the attempts to describe the genetic variability of the Scots pine indicates that the pine within its compact range is characterized by constant (clinal) variability, and that only isolated populations, such as the Scottish, Spanish, Balkan, Turkish or Caucasian populations display specific traits, not found in the described constant variability. Several attempts at describing Scots pine's genetic variability with a greater scope have been made, e.g. Svoboda (1953) or Molotkov and Patlay (1991). The results however are vastly heterogeneous (Giertych 1980; Przybylski 1970) and the only information that can be used in practice is the population-level data.

The knowledge of variability of a single population is furnished, among others, by provenance research. The first such experiment has been established in 1820 by Vilmorin. Since then, many provenance experiments for the Scots pine have been conducted, most of them in Poland, within the scope of the IUFRO research (Giertych and Oleksyn 1981). Data from provenance research so far, enabled us to distinguish populations characterized by excellent growth and quality traits. The group comprises: Rychtal, Gubin, Bolewice, Dłużek, Lipowa, Jegiel and Milicz. Relatively greater adaptation capabilities – plasticity – is the characteristic of the following populations: Bolewice, Dłużek, Milicz, Czersk, Goleniów, Parciaki, Prószków, Maskulińskie i Suchedniów. A significant level of adaptation to weaker and drier conditions is displayed in the populations of Rychtal, Lipowa and Supraśl, while populations adapted to richer and moister soils are: Jegiel, Janów Lubelski and Spała. In sub-mountainous conditions, in case of lack of own seed base, it is possible to use lowland pine populations of Lipowa, Supraśl, Janów Lubelski and Rozpuda (Matras 1994; Kowalczyk et al. 2000, 2006).

The results of research on Scots pine variability have been used i.a. in seed regionalization determining the rules of using pine's seed base. In the scope of the species' natural range in Poland, 25 maternal regions of provenance have been distinguished. Within a single origin region, only its own seed base may be used. The regions comprise populations that, based on provenance

research so far, are characterized by very good growth rate, plasticity and quality. They are, i.a. taborska pine – 106, napiwodzko-ramucka pine – 205, piska pine – 206, augustowska pine – 204, supraślaska pine – 207, tucholska pine – 305, bolewicka pine – 308, rychtalska pine – 501, spalska pine – 601, kozienicka pine – 602, parczewska pine – 404, as well as populations of an above average breeding value, growing in areas characterized by poor quality of pines, e.g. łochowska pine – 403, goleniowska pine – 101, and bytowska pine – 105.

There is significantly less information about genetic variability of the pine, based on molecular examination. In terms of genetic variation, the populations from maternal regions have been analyzed by Nowakowska (2007). Nowakowska proved that the highest variation in pine stands exists in the Baltic region, and the lowest – in Silesia, whereas the most homogeneous populations grow in the region of Mazury and Podlasie. Nowakowska's (2007) research confirms the significant impact of human economic activity on the shaping of tree stands, especially in the Silesian and Greater Poland/Pomeranian regions. Nowakowska also described the presence of the "b" haplotype of the *nadl* gene in Polish populations, a type that is typical for Spanish pine populations. In the light of Prus-Głowacki's et al. research (2012), this could be interpreted as the result of uncontrolled exchanges of seeds in 19th and early 20th centuries. Nowakowska (2007) did not prove any significant genetic diversity between the examined populations; she merely distinguished the Białowieża population as significantly different from the rest. Based on these results, Kowalczyk (2014) formulated a general conclusion, according to which, the nucleic SSR markers are inadequate for genetic variability comparison between populations. This paper attempts to fill in the missing genetic isoenzyme polymorphism information for the majority of the populations examined by Nowakowska (2007).

Isoenzyme polymorphism research has been conducted previously by Krzakowa et al. (1977). It described changes in 12 enzymatic loci in maternal trees and detected heterozygosity of 35%. Polish isoenzyme research has also been conducted by: Mejnartowicz (1979), Mejnartowicz and Bergmann (1985), Prus-Głowacki (1982), Prus-Głowacki and Nowak-Bzowy (1992), Prus-Głowacki and Bernard (1994), and Działuk and Burczyk (2002 and 2006). Results obtained in these

studies fail to provide a full answer to questions about genetic resources of pine in regions of provenance. That is why the formulated goal of the present research had been the presentation of the variation of the species in maternal regions of provenance: 107 (Międzyzdroje), 305 (Woziwoda), 206 (Strzałowo), 208 (Białowieża), 504 (Bolesławiec) and 606 (Józefów) (fig. 1).

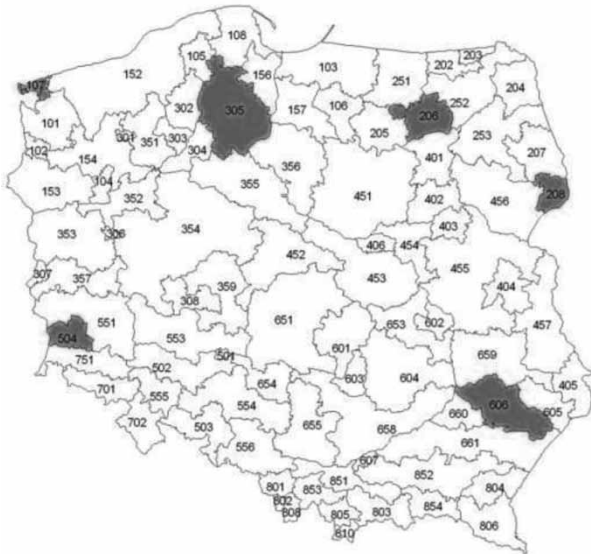


Figure 1. Geographic location of the six selected maternal regions of provenance

MATERIAL AND METHODS

The plant material used in the laboratory analysis has been collected in 6 maternal regions of provenance (fig. 1): 107 (Międzyzdroje), 305 (Woziwoda), 206 (Strzałowo), 208 (Białowieża), 504 (Bolesławiec), and 606 (Józefów). Genetic analyses constituted a supplement to project work. In each tree stand, the plant material has been collected from 30 randomly selected, standing trees. Tested trees were at least 30 meters away from the previous tree.

The collected plant material (stems with vegetative buds) has been kept in freezers, at a constant temperature of -24°C until the laboratory analysis. Before commencing electrophoresis, proteins from dormant and coverless vegetative buds have been isolated. The plant material has been homogenized, then extraction of proteins has been performed using 150 μl of extraction

buffer: 100 mM Tris-HCl pH 7.5 with the additions of 10mM 2-mercaptoethanol and 3 g PVP 25 per 100 ml of the buffer (Cieślewicz 2009). The obtained solution was used to soak strips of Whatman paper (31ET 4 X 11 mm) kept in freezers until the analysis.

The electrophoretic separation of proteins has been conducted in 13% starch gel (Starch-Art), using two buffer systems: A (184mM of boric acid, 38 mM of lithium hydroxide as pH buffer (pH 8.3) and 46 mM Tris, 7 mM of citric acid, 18mM of boric acid, 4 mM of lithium hydroxide as gel buffer (pH 8.5)) and C (127 mM Tris, 44 mM of citric acid as a pH buffer (pH 7.0) and 3.5 mM DL-histidine HCl, brought to 0.5 M Tris to pH 7.0 as pH buffer). Buffers used in the experiment are the same as in the following papers: Odrzykoski (2002) and Cieślewicz (2009).

Upon the completion of electrophoresis, the gel has been cut into layers 1.5 mm thick. Each of the layers has been then used to visualize the examined isoenzymes. The process has been conducted conforming to Concle et. al (1982) methodology. The following loci have been analyzed: *Gdh* (E.C.1.4.1.2), *Sdh-A Sdh-B* (E.C.1.1.1.25), *Pgd-B* (E.C.1.1.1.44), *Mdh-A, Mdh-C* (E.C.1.1.1.37), *Got-A, Got-B, Got-C* (E.C.2.6.1.1), *Dia-C* (E.C.1.8.1.4). The selected set of allozymes contained variable and monomorphic proteins.

Upon the completion of laboratory analysis, genetic variability parameters of the examined populations have been estimated, using specialized statistics packages: Gen Alex 6.1 (Peakal and Smouse 2006) and PopGen 1.32 (Yeh and Boyle 1997). Genetic variability parameters have been examined, i.a.: average number of alleles per locus (N_a), effective number of alleles per locus (N_e), observed heterozygosity (H_o) (Nei 1973), expected heterozygosity (H_e), as well as the inbreeding coefficient F (Hartl and Clark 2007).

RESULTS

The results of the conducted research on allele frequency and rare alleles have been presented in table 1, whereas genetic variability parameters of the examined populations have been presented in table 2. Figure 2 shows the diversity between various tree stands. Based on the collected data, it has been concluded that there are 28 alleles per population on average, while the av-

Table 1. Frequency of alleles of studied populations (rare alleles in gray)

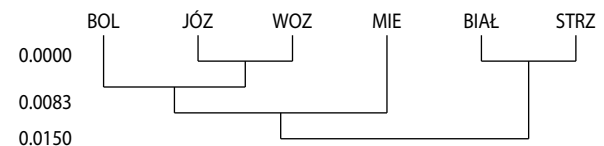
Locus	Allele	Strzałowo	Bolesławiec	Białowieża	Józefów	Międzyzdroje	Woziwoda
<i>GOT-A</i>	1	0.90	0.89	0.90	0.98	0.97	0.97
<i>GOT-A</i>	3	0.10	0.11	0.10	0.02	0.03	0.03
<i>GOT-B</i>	1	0.35	0.57	0.40	0.48	0.52	0.44
<i>GOT-B</i>	2	0.65	0.43	0.60	0.52	0.48	0.56
<i>GOT-C</i>	1	0.29	0.32	0.36	0.35	0.50	0.37
<i>GOT-C</i>	2	0.71	0.68	0.64	0.65	0.50	0.63
<i>DIA</i>	1	0.65	0.84	0.77	0.91	0.94	1.00
<i>DIA</i>	2	0.35	0.16	0.23	0.09	0.03	0.00
<i>DIA</i>	4	0.00	0.00	0.00	0.00	0.03	0.00
<i>GDH</i>	1	0.54	0.32	0.48	0.40	0.54	0.44
<i>GDH</i>	2	0.46	0.68	0.52	0.60	0.46	0.56
<i>MDH-A</i>	1	0.94	1.00	0.85	0.98	0.92	0.95
<i>MDH-A</i>	2	0.06	0.00	0.15	0.02	0.08	0.05
<i>MDH-C</i>	1	0.66	0.60	0.62	0.58	0.71	0.64
<i>MDH-C</i>	2	0.34	0.40	0.38	0.42	0.29	0.36
<i>SDH-A</i>	1	0.97	0.92	0.98	0.98	1.00	0.97
<i>SDH-A</i>	2	0.03	0.08	0.02	0.02	0.00	0.03
<i>SDH-B</i>	1	0.87	0.94	0.92	0.90	0.98	0.86
<i>SDH-B</i>	2	0.13	0.06	0.08	0.05	0.00	0.14
<i>SDH-B</i>	3	0.00	0.00	0.00	0.05	0.02	0.00
<i>PGD</i>	1	0.77	0.80	0.79	0.89	0.95	0.85
<i>PGD</i>	2	0.23	0.18	0.21	0.11	0.05	0.15
<i>PGD</i>	4	0.00	0.02	0.00	0.00	0.00	0.00

average quantity of alleles in a locus equals 1.98. Populations differ between one another in allele frequency. Statistically significant differences in allele frequency have been stated in 10 populations: Strzałowo (*Got-C*), Bolesławiec (*Got-B*), Białowieża (*Got-B*, *Gdh*), Józefów (*Mdh-C*), Międzyzdroje (*Got-B*, *Got-C*, *Mdh-C*), and Woziwoda (*Got-C*, *Gdh*). The remaining alleles showed no significant differences. By analyzing the significantly different alleles, it has been observed that the following alleles are dominant for their respective population(s):

Strzałowo *Got-C-2*, (71%), Bolesławiec *Got-B-1*, (57%), Białowieża *Got-B-2* (60%) and *Gdh-2* (52%), Józefów *Mdh-C-1* (58%) i *Mdh-C-1* (71%), Woziwoda *Got-C-2* (63%) and *Gdh-2* (55%), Międzyzdroje *Got-B-1* (51%), while *Got-C-1* and *Got-C-2* had an equal frequency of (50%) (see tab. 1).

Table 2. Effective number of alleles per locus (N_e), heterozygosity: expected (H_e) and observed (H_o), inbreeding coefficient (F) in examined populations

		N_e	H_o	H_e	F
Strzałowo		1.54	0.35	0.32	-0.09
	SE	0.11	0.07	0.05	0.09
Bolesławiec		1.48	0.26	0.29	0.07
	SE	0.11	0.05	0.05	0.07
Białowieża		1.55	0.25	0.32	0.12
	SE	0.11	0.03	0.05	0.07
Józefów		1.45	0.27	0.26	-0.04
	SE	0.13	0.08	0.06	0.07
Międzyzdroje		1.42	0.23	0.24	-0.02
	SE	0.14	0.07	0.07	0.08
Woziwoda		1.36	0.22	0.26	0.07
	SE	0.19	0.05	0.06	0.07

**Figure 2.** Dendrogram of genetic distance, according to Nei (1987) of studied populations

Rare alleles (frequency below 5%) have been found in all examined populations. The frequency of rare alleles has usually oscillated between 1.6 to 3.2%. Allele 2 in the *Sdh-A* locus has been the most common rare allele in the examined populations. Its presence has been detected in: Strzałowo, Białowieża, Józefów and Woziwoda. Rare alleles that could constitute population markers have also been described. Among them is the diaphoresis allele 4, present exclusively in the Międzyzdroje tree stand.

In the analyzed tree stands, the average effective number of alleles per locus amounted to 1.46 and had

been inferior to the expected per locus allele quantity of 1.93. Specific populations, the effective number of alleles per locus has been: 1.54 in Strzałowo and Białowieża, 1.48 in Bolesławiec, 1.44 in Józefów, 1.42 in Międzyzdroje and 1.35 in Woziwoda (tab. 2).

Average observed heterozygosity in examined populations equalled 0.26 and was inferior to expected heterozygosity by 0.02. Specific populations had the following values of heterozygosity: Strzałowo 0.35, Bolesławiec and Białowieża 0.25, Józefów 0.27, Międzyzdroje 0.23, Woziwoda 0.26. Specific loci had different levels of heterozygosity, the highest noted in *Mdh-C*, and the lowest – *Got-C* (tab. 2).

The estimated level of inbreeding has divided the examined populations into two groups – one, where the *F* coefficient was positive, and the other, where it was negative. The first group comprises the populations of: Białowieża (0.12), Woziwoda (0.07) and Bolesławiec (0.07), the other: Strzałowo (–0.09), Józefów (–0.03) and Międzyzdroje (–0.01). In the six examined populations, the genetic differentiation coefficient (Nei 1973) equalled 7.7%.

In order to determine the genetic differentiation, genetic distances between the examined populations have been calculated. The dendrogram divided the populations into two groups (fig. 2). The first group included the Białowieża and Strzałowo populations, the remaining four populations constituted the other group. Two populations are the most similar to one another, based on Nei's (1973) genetic differentiation coefficient, namely Woziwoda and Józefów. Their genetic distance equals 0.0032. Międzyzdroje however, is the population with the greatest genetic distance in the second subgroup, namely 0.0094.

Through analysis of genetic variability parameters, the examined populations have been divided into two sub-groups, based on their inbreeding coefficient F_{is} . The first subgroup comprises populations with a greater heterozygosity, than the expected one, according to Hardy Weinberg law: Międzyzdroje, Strzałowo and Józefów. The second subgroup contains populations where homozygotes are dominant: Białowieża, Woziwoda and Bolesławiec. Particular attention has been granted the Białowieża population, whose inbreeding coefficient F_{is} equals 0.12. The high value of inbreeding in Białowieża results from homozygosity of two loci: *Got-B* and *Gdh*.

DISCUSSION

In 2000, in Poland, the boundaries of regions of provenance of basic reproduction material (Forest Reproductive Material – FRM) for 10 forest-forming tree species have been established. These regions have been created on the basis of ecological differentiation, based on forest regionalization, as well as information obtained from provenance and genetic research (Matras 1996; Resolution of the Minister of Environment of 9th March 2004; Nowakowska and Rakowski 2005). Originally, the region boundaries have been established on the basis of observed phenotypic traits in examined tree populations (Matras 2005; Matras et al. 1993; Nowakowska and Rakowski 2005). It is therefore important to determine the true genetic variability of seed regions in Poland, based on actual genetic research.

Forest trees belong to the most variable organisms in nature. It is in part due to the size of their genome. For example, the pine comprises about 50 billions nucleotide pairs (Paule 1992). It has also been observed that generations of trees may pollinate one another, which makes it all the more difficult to determine genetic differentiation of the population. As a consequence, it is possible for pine populations that are very remote geographically and different phenotypically to display close genetic resemblance (Krzakowa 1979; Matras 2005).

Scots pine diversity in Poland is clinal, and its main source is the inter-population variability. Nowakowska and Rakowski (2005) and Nowakowska (2007) indicate that as much as 70% of genetic variability of Scots pine is due to inter-population variability. This is also corroborated by the present study, in which all analyzed populations have been characterized by a greater inter-population diversity, than between populations ($F_{st} = 0.077$). It is therefore justified to put forth a general thesis that it is crucial for forestry to preserve economically valuable tree stands, as they most likely contain the unique gene sets for their respective areas.

The average allele quantity per locus in the present research has been determined at 1.98. This result is significantly lower than in Prus-Głowacki and Bernard's research of 1994, or in those of Samoćko (2004) and Działuk et al. (2006). The lower allele quantity per locus is most likely due to using a set of less polymorphic loci, than in the abovementioned publications.

In the present work, the populations with the greatest allele quantity per locus were: Białowieża and Strzałowo. These populations are characterized by a high heterozygosity coefficient. In addition, tree stands from Strzałowo and Białowieża constitute a separate group on the dendrogram, which is indicative of their genetic autonomy. This result could suggest their indigeness. Based on the results, it could be deduced with high probability of success that the influence of uncontrolled seed exchanges on the genetic structure of Białowieża and Strzałowo populations has been insignificant. Results of other research, i.a. that of Nowakowska (2007) and historic information indicate that Polish tree stands, especially in the Prussian territory from the Partition era in the 19th century have been over-exploited. Seeds for forestation purposes have been obtained from various, often situated far from places of origin. The present research confirms the influence of over-exploitation of tree stands in western Poland, which is consistent with earlier research. The Międzyzdroje population is particularly interesting, where diaphoresis allele 4 and Phosphoglucosomerase allele 4 have been found. These are rare alleles, and in the scope of the present research, they have only been found in this population. It is supposed that these proteins could be a trace of dominant alleles in a different climatic region, which constitutes proof of the foreign origin of the seeds, from which the examined tree stand has been bred.

To sum up, it is interesting to note that genetic variability of Scots pine in Poland, based on isoenzyme research has for the most part confirmed the results of other molecular research and breeding experiments. It is therefore correct to assume that the distinction of seed regions and other actions aiming to preserve their gene resources are fully justified.

CONCLUSIONS

1. Molecular research using isoenzyme markers has confirmed the diversity of Scots pine, based on breeding traits and the fact of significantly higher intra-population genetic variability within the species.
2. Two of the examined populations: Strzałowo and Białowieża are characterized by an individual and distinct genetic structure, which is was shown on dendrogram configuration and the highest value effective number of alleles values. For this reason, they should be the first to be included in Scots pine genetic variability protection programs, executed by Polish State Forests National Forest Holding.
3. Rare forms of unique alleles found for Scots pine populations of West Poland e.g. from Międzyzdroje can confirm the influence of forest over-exploitation and uncontrolled seed exchanges of the 19th century in western Poland.

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