Ploidy level and breeding system in some populations of *Pilosella* (Asteraceae) in eastern and southern Slovakia

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Abstract: The ploidy level/breeding system was determined in following species and hybrids originating from populations of the agamic polyploid complex of *Pilosella* in Slovakia: *P. bauhini* (either sexual tetraploids or apomictic pentaploids), *P. hoppeana* subsp. *testimonialis*, *P. lactucella*, *P. onegensis* (all three taxa diploid and so supposedly sexual), *P. officinarum* (pentaploids, hexaploids and octoploids, all cytotypes apomictic), *P. glomerata* (one pentaploid, another plant hexaploid and apomictic), *P. macrostolona* (apomictic hexaploids), *P. schultesii*, (mostly tetraploid, one plant an apomictic pentaploid), *P. lactucella* × *P. onegensis* (diploid and sexual), *P. lactucella* × *P. aurantiaca* (triploid and apomictic) and *P. bauhini* × *P. officinarum* (both sexual and apomictic tetraploids, apomictic pentaploids and apomictic hexaploids). The paper provides two karyological novelties in *Pilosella*: (a) A new hexaploid cytotype was revealed in *Pilosella glomerata*; (b) The octoploid apomictic and monoclonal plants of *Pilosella officinarum* were grown from seeds suggesting an occurrence of fruiting octoploid maternal plant(s). Such a cytotype would represent a new highest ploidy level detected in *P. officinarum* in the field. The cytotypes that were attributed both to *P. officinarum* and to the hybrids of *P. bauhini* and *P. officinarum* differed in a within-population clonal diversity. This effect could result from a different impact of (residual) sexuality and/or a different rate of origin of particular hybrid cytotypes. All findings presented in the paper are compared with published data on *Pilosella* species that refer preferentially to Slovakia, but also to a broader area in Central Europe.

Keywords: hawkweed, chromosome number, DNA-ploidy level, facultative apomixis, clonal diversity, Slovenský raj, Cerová vrchovina, Krupinská planina.

Introduction

Taxonomic complexity in *Pilosella* Hill, formerly placed in the genus *Hieracium* L. s.l., has following reasons (Fehrer et al. 2007): (a) diversity in ploidy level (the basic chromosome number x = 9, diploids to octoploids occur in the field), (b) diverse breeding systems (sexual or facultatively apomictic, the sterile plants are also occasionally found), and (c) both past and recent hybridization. The current Flora of Slovakia comprises 31 species of *Pilosella* (Marhold & Hindák 1998; Marhold et al. 2007), but an extent of exploration of karyological and reproductive characters varies among individual species. Specifically, the cytotype variation has most intensively been studied in *Pilosella officinarum* F. W. Schultz & Sch. Bip. that is abundant in Central Europe including Slovakia (Mráz et al. 2008). *Pilosella bauhini* (Schult.) Arv.-Touv. has also been studied thoroughly in Slovakia, especially with respect to variation in ploidy level and reproduction mode (Rotreklová 2004; Rotreklová et al. 2005). Similarly to *Pilosella officinarum*, a contrasting pattern of ploidy distribution in Central Europe has also been found in *P. bauhini* (Rotreklová 2004; Rotreklová et al. 2005). The *Pilosella alpicola* group that is confined to alpine areas, is the third group of taxa that had systematically been explored in Slovakia, including the variation in ploidy level and breeding system (Šingliarová & Mráz 2009; Šingliarová et al. 2011). Finally, both the ploidy level and breeding system were studied in five populations of *Pilosella echioides* (Lumn.) F. W. Schultz & Sch. Bip. in Slovakia (Peckert et al. 2005; Rotreklová et al. 2005). The karyological and/or reproductive attributes of the other species of *Pilosella* were occasionally studied in Slovakia and were published elsewhere. The Karyological database of ferns and flowering plants of Slovakia (Marhold et al. 2007) overviews the data on altogether 20 *Pilosella* species and hybrids that were published before 2007. However, one of the species in that database, *Pilosella decolorans* (Fr.) F. W. Schultz & Sch. Bip., is currently classified at subspecific level and it is placed in the species of *Pilosella aurantiaca* (L.) F. W. Schultz & Sch. Bip. (Bräutigam & Greuter 2007–2009+). Our present study contributes to the knowledge about ploidy level/chromosome number in altogether twelve taxa and hybrids of *Pilosella* originating from eastern and southern Slovakia; out of them, the breeding system was examined in seven taxa and three hybrids (Tab. 1). A within-population clonal (genotype) diversity was examined in particular species/hybrids originating from altogether six localities (Tab. 2). Hereby we aimed at the question, to a what extent is the sexual/apomictic reproduction in particular cytotypes reflected in the clonal diversity assessed by pattern of isozyme phenotypes.

Material and Methods

Plant material

The plants were sampled during excursions to the Slovenský raj Mts., the Krupinská planina plateau and the Cerová vrchovina Highland. The sampling excursions were realized within 8th *Hieracium* Workshop (held in 2004 in Dedinky, Slovenský raj),

within the projects performed in the Institute of Botany of the Czech Academy of Sciences, Průhonice (excursions in 2008, 2011 and 2012), and occasionally also during private trips (plants collected by F. Krahulec in 2000 and 2005). The individual localities of *Pilosella* plants sampled (Fig. 1), completed by a list of taxa and cytotypes recorded, are described in detail below. According to phytogeographical division of Slovakia (Futák 1980), the localities in the Slovenský raj Mts. belong to the phytogeographical district no. 17 "Slovenský raj", while the localities in the Krupinská planina plateau and the Cerová vrchovina Highland belong to the phytogeographical district no. 2 "Ipeľsko-rimavská brázda". The phytogeographical division of Slovakia is also illustrated in the map available in the Karyological database of ferns and flowering plants of Slovakia (Marhold et al. 2007).

Collecting the living plants in the field, we especially focused either on those populations of *Pilosella* that were rich in species, hybrids and morphotypes, or on the individual plants with specific morphological characters suggesting an uncommon hybridogenous species or recent hybrid. In the species-rich populations we accepted the same approach as we used previously in other countries in Central (e.g. Krahulec & Krahulcová 2006; Krahulcová et al. 2012, 2014) and southeastern Europe (Krahulcová et al. 2018). Treating the whole species-rich population as one entity, we sampled all morphologically distinguishable mature plants and transported them to the experimental garden of the Institute of Botany, Průhonice (Czech Republic) in order to carry out subsequent treatments: the determination of ploidy level/chromosome number, the breeding system, and the detection of the clonal structure, if needed. The taxonomic identity of all *Pilosella* plants was determined in the field by F. Krahulec; in addition, the plants collected in 2004 were determined in cooperation with *Hieracium*-specialists attending the 8th *Hieracium* Workshop (Siegfried Bräutigam, Franz Schuhwerk, Jindřich Chrtek, Patrik Mráz and others). Using the herbarium specimens of cultivated plants, the taxonomic identity was definitely determined/revised by František Krahulec, Jindřich Chrtek (Institute of Botany, Průhonice) and Siegfried Bräutigam (Dresden, Germany). The plant names follow Bräutigam & Greuter (2007–2009+). Voucher specimens of all plants studied are deposited in the Herbarium of the Institute of Botany, Průhonice (PRA).

Detection of ploidy level, chromosome number, breeding system and pollen stainability

Using flow cytometry (FCM), we determined DNA ploidy level in all plants cultivated in the experimental garden. The DAPI staining method (Otto 1990) was applied using the nuclei-extracting buffer (Otto I) and the staining buffer (Otto II) supplemented with mercaptoethanol (2 µl ml-1) as an antioxidant. Fluorescence intensity was determined using either a CyFlow Cytometer or a PA II Ploidy Analyser Partec GmbH, Münster, Germany), both equipped with an HBO high–pressure mercury lamp for UV excitation. For details of the procedure, see Krahulcová et al. (2004). Relative DNA content was estimated using the diploid *Pilosella lactucella* as an internal standard (DNA content 4.07 pg/2C, Suda et al. 2007). When evaluating

the FCM analyses, only those histograms in which the coefficient of variance of peaks did not exceed 3% were accepted. A total of 3000 nuclei were scored for each sample. In addition, chromosomes were counted in some plants using the squashed root-tip meristems stained with lacto-propionic orcein (Dyer 1963).

The breeding system (sexual versus apomictic reproduction mode) was principally determined comparing the seed-set in the emasculated versus in the open-pollinated plants (e.g. Gadella 1984). This method detects a parthenogenetic seed development and it is routinely used in Compositae with an autonomous apomixis (Richards 1986). The fertilization-independent seed formation is predominantly coupled with apomeiosis in wild-type apomicts within *Pilosella* (Bicknell & Koltunow 2004) and therefore the emasculation test is an approximation for apomictically reproducing plants (Hand et al. 2015). The seed-set was not quantified in examined plants. The presence of well-developed (filled) achenes in each of three emasculated capitula per plant (checked by a fine pressure on achenes using the stereomicroscope and tweezers) was sufficient for determination of apomictic reproduction mode. Those plants that were scored as sexual, formed a normal seed-set in open pollinated capitula, whereas the emasculated capitula did not form seeds at all. In some cases, Flow Cytometric Screen of Seeds was used for the determination of reproductive origins of seeds in the respective maternal plant (FCSS method, Matzk et al. 2000). The seed doublets were analysed together with an internal standard in each sample (for details of the FCSS procedure modified for *Pilosella*, see for example Krahulcová et al. 2014, 2018). The third supporting criterion for the determination of the sexual versus apomictic reproductive mode was an assessment of ploidal diversity in the progeny arrays in relation to ploidy of the respective maternal plant. Generally, the heteroploid hybrids are expected among the progeny originating from the sexual maternal plants in the mixed-ploidy populations of *Pilosella*, whereas the maternal plants with a high penetrance of apomixis produce primarily the progeny maintaining the maternal ploidy level, in addition to polyhaploids (Bicknell & Koltunow 2004; Hand et al. 2015; Krahulcová et al. 2014, 2018; Rosenbaumová & Krahulec 2015). Two methods were used for the progeny ploidy screening. (A) The seeds that originated from open-pollinated plants were pooled (six to ten seeds per sample) and analysed using the modified FCSS method (Krahulcová et al. 2014, 2018). This procedure allows to screen and quantify the embryos according to ploidy level (Krahulcová & Suda 2006). (B) The progeny were grown from remaining seeds that were sampled in cytotype-mixed populations. This progeny that were cultivated in experimental garden to maturity, were used for another follow-up study assessing the mother-progeny relationships, principally with respect to karyological diversity. The use of particular methods for the determination of breeding system in individual plants is given in Tab. 3.

The pollen stainability, that is indicative of pollen fertility, was examined in selected plants in order to support/reject the hypothesis about hybrid origin. The pollen from intact (unruptured) anthers was prepared from three inflorescences (capitula) per plant and stained by either Alexander's stain (using fresh pollen) or

Tab. 1 Karyological and reproductive attributes of *Pilosella* taxa included in this study.

Species/hybrid	DNA ploidy level	Reproduction mode
P. bauhini subsp. *bauhini*	5x	apomictic
P. bauhini subsp. *magyarica* (Peter) S. Bräut.	4x	sexual
P. hoppeana subsp. *testimonialis* (Peter) P.D. Sell et C. West	2x	n.d.
P. lactucella (Wallr.) P.D. Sell et C. West	2x	sexual
P. officinarum F. W. Schultz et Sch.-Bip.	5x	apomictic
	6x	apomictic
	*8x	*apomictic
P. onegensis Norrl.	2x	n.d.
P. glomerata (Froel.) Fr.	5x	n.d.
	6x	apomictic
P. macrostolona (Gus. Schneider) Soják	6x	apomictic
P. schultesii (F.W. Schultz) F.W. Schultz et Sch. Bip.	4x	n.d.
	5x	apomictic
P. lactucella × *P. onegensis*	2x	sexual
P. lactucella × *P. aurantiaca*	3x	apomictic
P. bauhini × *P. officinarum*, including *P.* × *brachiata* (DC.) F.W. Schultz et Sch. Bip.	4x	sexual
	4x	apomictic
	5x	apomictic
	6x	apomictic

Abbreviation: n.d. – not determined. *cultivated progeny were grown from seeds that were sampled from several plants of *P. officinarum* (with unknown ploidy level) in the field.
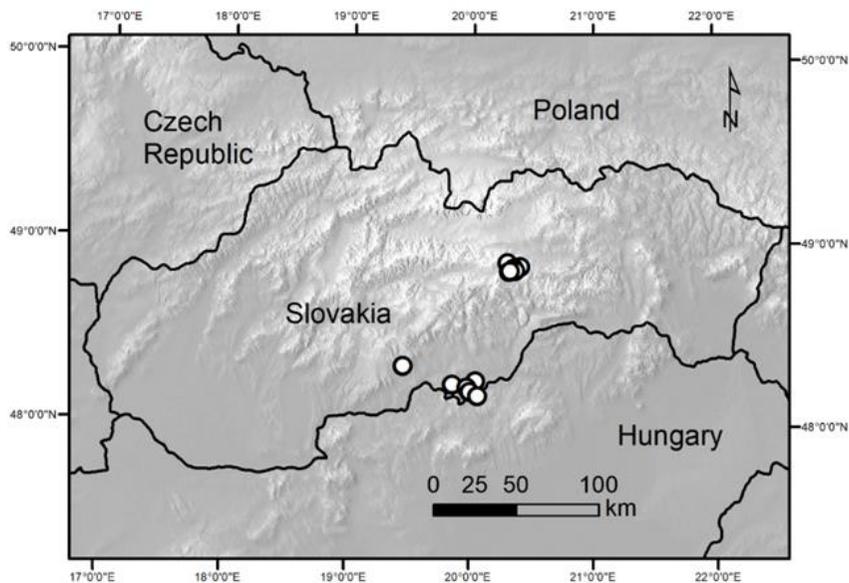
Fig. 1. Map of Slovakia with studied localities (orig. J. Wild).

cotton-blue stain in lactophenol (using fixed pollen). The procedure of pollen staining followed Rotreklová & Krahulcová (2016), and the stainability was evaluated in 400 pollen grains in each capitulum.

Clonal structure

A common facultative apomixis and clonal growth by means of stolons (e.g., Krahulcová et al. 2000, 2009) shape the clonal structure of *Pilosella* populations, thus stimulating the spread of conserved genotypes. The vegetative spread through stolons is effective in a rather small area surrounding the respective source plant in a population. Along with a vegetative spread, the clonal apomictic seeds from a single maternal individual may spread further to neighbouring populations. Assessing the clonal structure of plants at selected localities, we evaluated separately the sub-groups of plants that differed from each other in morphological traits, cytotype and reproduction mode, whereas these attributes were homogeneous within each sub-group (Tab. 2). A minimum number of individual genotypes within each sub-group was inferred from a unique pattern of isozyme phenotypes when combining four enzymes tested (AAT, LAP, 6-PGDH/PGM and EST). For a description of methodology of isozyme analysis see Krahulec et al. (2004). In previous studies, these systems provided sufficient resolution within individual cytotypes of numerous polyploid taxa of *Pilosella* (Krahulcová et al. 2009, 2012, 2014).

Taxonomic concept in *Pilosella*

We accept the species and hybrid concept corresponding to classification by Bräutigam & Greuter (2007–2009+) and Bräutigam (2017). Nevertheless, the problem in numerous hybrids of *Pilosella* is in unfeasible distinguishing between stabilized hybridogenous types (recognized as species) and recent hybrids of the same putative origin. Both the recent and stabilized hybrids combine the morphological characters of their putative parents. Origin of stabilized hybridogenous species in *Pilosella* is ordinarily represented by a hybrid formula illustrating the balance/prevalence of morphological characters of parental species (e.g. Bräutigam 2017). We use the hybrid formulae also here in Results, namely, in square brackets below the name of the respective hybridogenous species. Those hybrids that usually co-occurred with both putative parents in hybridizing populations we consider to be recent (e.g. Krahulcová et al. 2014): therefore we use for them the combination of putative parental species with the symbol '×'.

Results

Localities and attributes of recorded taxa

National park Slovenský raj:

1. Kopanisko, on the meadow margin close to roadside, 980 m a.s.l., 48°54'00"N, 20°17'35"E, coll. F. Krahulec, 18 June 2004.

Recorded hybrid: *P. macrostolona* or alternatively *P. officinarum × P.onegensis* (2n ~ 6x, apomictic: plant 927).

2. Dedinky, the plateau Geravy, meadow along the hiking path, 1037 m a.s.l., 48°52'53"N, 20°23'41"E, coll. F. Krahulec, 18 June 2004.

Recorded hybrids: *P. lactucella × P. aurantiaca* (2n ~ 3x, apomictic: plant 929).

The plant resembling the hybridogenous species of *P. floribunda* and possibly corresponding to hybrid *P. onegensis × P. lactucella*, was also recorded at the locality; this plant was only sampled for a herbarium specimen.

3. Stratená, surroundings of the village, 870 m a.s.l., 48°52'20"N, 20°20'40"E, coll. F. Schuhwerk, 17 June 2004.

Recorded hybrid: *P. schultesii* (2n ~ 4x: plant 928).

4. Stratená, meadows near Stratenská Píla settlement, along the hiking path between the villages Dedinky and Stratená, 795 m a.s.l., 48°51'30"N, 20°21'30"E, coll. F. Krahulec, 19 June 2004.

Recorded hybridogenous species: *P. glomerata* (2n ~ 5x: plant 919; 2n = 6x = 54, apomictic: plant 920).

The basic species of *P. caespitosa* was also recorded at the locality, but this plant was only sampled for a herbarium specimen.

5. Stratená, the meadow area "Pod Hanisejom" and meadows between this area and the area "Voniarky", 900–910 m a.s.l., 48°51'15"–48°51'26"N, 20°18'23"–20°18'43"E, coll. F. Krahulec, 19 June 2004 and F. Krahulec, A. Leskovjanská, T. Urfus and J. Chrtek, 5 July 2008.

Recorded basic species: *P. lactucella* (2n ~ 2x, sexual: plants 1544 and 1546A; 2n = 2x = 18, sexual: plant 917); *P. onegensis* (2n ~ 2x: plants 1541A and 1541B); *P. officinarum* (2n ~ 6x, apomictic: plants 1542 and 1551; 2n=6x=54, apomictic: plant 1548); *P. bauhini* subsp. *bauhini* (2n ~ 5x, apomictic: plants 1545A and 1546B; 2n = 5x = 45, apomictic: 1545B);

Hybrids: *P. schultesii* (2n ~ 4x: plant 923); *P. schultesii* or alternatively *P. piloselliflora* (2n ~ 4x, apomictic: plant 930); *P. macrostolona* or alternatively *P. officinarum × P. onegensis* (2n ~ 6x, apomictic: plant 918); *P. lactucella × P. onegensis* (2n ~ 2x, sexual: plant 924); *P. lactucella × P. aurantiaca* (2n ~ 3x, apomictic: plants 922 and 1538B; 2n = 3x = 27, apomictic: plants 925 and 1539); *P. brachiata* (2n ~ 5x, apomictic: plant 1547); *P. bauhini × P. officinarum* (2n ~ 5x, apomictic: plant 1550); *P. bauhini ×* unknown parent (2n ~ 5x, apomictic: plant 1549); *P. officinarum ×* unknown parent (2n ~ 4x, apomictic: plant 1543);

Unclassified hybrids of unknown origin: *Pilosella* sp. (2n ~ 4x: plant 1540; 2n ~ 5x, apomictic: plant 921). The tetraploid hybrid might have arisen from a cross between the hexaploid *P. officinarum* and the diploid *P. lactucella* that both co-occur at the locality. The stabilized hybrids of this parentage are commonly classified as *P. schultesii* (e.g. Bräutigam 2017).

In the list of plants recorded at this locality we included four undetermined hybrids (plants 1549, 1543, 1540 and 921) as indication of their occurrence there; these mostly apomictic plants with determined ploidy level are documented by herbarium specimens as other plants studied.

Krupinská planina plateau:

6. distr. Veľký Krtíš, Pravica village: along the hiking path (red sign), ca. 0.75–1 km N of the village of Pravica, ca. 14 km W of the town of Lučenec, 390 m a.s.l., 48°19'54.2''N, 19°26'43.0''E, coll. F. Krahulec, T. Urfus and A. Krahulcová, 26 May 2011, F. Krahulec and T. Urfus, 31 July 2012.

Recorded basic species: *P. bauhini* subsp. *magyarica* (2n ~ 4x, sexual: plants 1972, 1978 and 2081); *P. bauhini* s. l. (2n ~ 4x: plants 2054, 2055 and 2083: all three plants without voucher documentation, sampled exclusively for the purpose of verification of tetraploid ploidy level); *P. officinarum* (2n = 5x = 45, apomictic: plant 1975; 2n ~ 5x: plants that were sampled exclusively for detection of the clonal structure 2035–2043, 2045–2048, 2050, 2053, 2056–2059, 2061, 2064–2074, 2078–2080, 2082, 2084–2089; 2n ~ 6x: plants 2044, 2049, 2051, 2052, 2075, 2076, 2077 in addition to 8x progeny grown from seeds that were sampled at the locality from several other fruiting plants);

Hybrid: *P. bauhini* × *P. officinarum* (2n ~ 4x, sexual: plants 1971, 1973 and 1974).

Cerová vrchovina highlands:

7. distr. Rimavská Sobota, Gemerské Dechtáre village: grassy slopes (loess) on the SE periphery of the village past the football ground, 234 m a.s.l., 48°15'18.9''N, 20°02'15.5''E, coll. F. Krahulec, 9 September 2005, F. Krahulec, T. Urfus and A. Krahulcová, 26 May 2011.

Recorded basic species: *P. bauhini* subsp. *magyarica* (2n ~ 4x, sexual: plants 1164, 1941, 1951, 1953 and 1955); *P. officinarum* (2n ~ 5x, apomictic: plants 1944 and 1945; 2n = 5x = 45, apomictic: plant 1954; 2n ~ 5x: plant 1165; 2n ~ 6x, apomictic: plant 1952); *P. lactucella* (2n ~ 2x: plant 1942);

Hybrids: *P. bauhini* × *P. officinarum* (2n ~ 4x, sexual: plants 1947 and 1950; 2n ~ 4x: plant 1947/2, monoclonal with sexual plant 1947; 2n ~ 5x, apomictic: plants 1946, 1948, 1948/2 and 1956, the last plant with a detectable residual sexuality); *P. schultesii* (2n ~ 5x, apomictic: plant 1943).

8. distr. Fiľakovo, Belina village: grassy slopes above the cemetery NEE of the village, 235 m a.s.l., 48°14'02.3''N, 19°51'00.2''E, coll. F. Krahulec, T. Urfus and A. Krahulcová, 25 May 2011.

Recorded basic species: *P. bauhini* subsp. *magyarica* (2n ~ 4x, sexual: plants 1925, 1926, 1937 and 1940); *P. hoppeana* subsp. *testimonialis* (2n ~ 2x: plant 1924); *P. officinarum* (2n = 5x = 45, apomictic: plant 1927; 2n ~ 5x, apomictic: plant 1928; 2n ~ 6x: 23 progeny plants that were grown from mixture of seeds sampled at the locality from several fruiting plants of *P. officinarum*);

Hybrid: *P. bauhini* × *P. officinarum* (2n ~ 5x, apomictic: plants 1930, 1931A, 1933, 1935 and 1936; 2n ~ 5x: plant 1934, monoclonal with apomictic plant 1933; 2n ~ 6x, apomictic: plants 1929 and 1931B; 2n ~ 6x: plant 1932, monoclonal with apomictic plants 1929 and 1931B).

9. distr. Rimavská Sobota, Hajnáčka village: eastern edge of the village along a hollow way past the cemetery, 274 m a.s.l., 48°13'10.5''N, 19°57'57.1''E, coll. F. Krahulec, 14 September 2000, F. Krahulec, T. Urfus and A. Krahulcová, 25 May 2011.

Recorded basic species: *P. bauhini* subsp. *magyarica* (2n = 4x = 36, sexual: plant 1966; 2n ~ 4x: plant 1970, monoclonal with sexual plant 1966); *P. officinarum* (2n ~ 5x: plant 1968);

Hybrids: *P. bauhini* × *P. officinarum* (2n ~ 4x, sexual: plant 1967; 2n ~ 5x, apomictic: plant 1969); *P. brachiata* (2n ~ 5x: plant 496/2).

10. distr. Rimavská Sobota: grassy slopes (loess) along the north margin of the road connecting the villages Dubno and Gemerský Jablonec, 238 m a.s.l., 48°11'46.9''N, 19°59'29.4''E, coll. F. Krahulec, T. Urfus and A. Krahulcová, 26 May 2011, F. Krahulec and A. Krahulcová, 28 April 2012.

Recorded basic species: *P. bauhini* subsp. *magyarica* (2n ~ 4x: plant 1959); *P. officinarum* (2n ~ 5x, apomictic: plant 2026; 2n ~ 6x, apomictic: plants 1960 and 2023; 2n ~ 6x: 2020–2022, 2024, 2025 and 2027);

Hybrid: *P. bauhini* × *P. officinarum* (2n ~ 4x, sexual: plants 1961, 1962/1, 1963 and 1964; 2n ~ 4x, apomictic: plants 1962/2 and 1962/3; 2n ~ 4x: plant 1965).

11. distr. Rimavská Sobota: Petrovce, along the hiking path following the Slovak – Hungarian border ca. 3 km SE of the village, ca. 300 m a.s.l., 48°10'22''N, 20°03'18''E, coll. F. Krahulec and A. Krahulcová, 24 April 2012.

Recorded basic species: *P. bauhini* subsp. *magyarica* (2n ~ 4x: plant 2011); *P. officinarum* (2n ~ 5x: plant 2015; 2n ~ 6x: plants 2009, 2013, 2016 and 2018);

Hybrid: *P. bauhini* × *P. officinarum* (2n ~ 5x, apomictic: plants 2010, 2012 and 2019; 2n ~ 5x: plant 2017).

Clonal structure in selected taxa and cytotypes

Each of the tetraploid plants of *P. bauhini* that were sampled within three localities (localities 6, 7 and 8), had a distinct isozyme phenotype (Tab. 2). The pentaploid and hexaploid *P. officinarum* was recorded at the locality 6, in addition to sampled seeds that gave rise to progeny constituting an octoploid apomictic clone. Although we did not sample at this locality any mature octoploid plant of *P. officinarum* for cultivation, we collected there a mixture of seeds from several fruiting plants of this species. All seven raised progeny plants that corresponded in morphology to *P. officinarum* were octoploid as inferred from FCM, and the counting of chromosomes confirmed the chromosome number of 2n = 72 in one of these plants. The all seven plants shared an identical isozyme phenotype (Tab. 2). The four hybrid and apomictic plants of *Pilosella lactucella* × *P. aurantiaca* at the locality 5 were not monoclonal, comprising altogether three isozyme phenotypes (Tab. 2). Clonal structure of the hybrids *Pilosella bauhini* × *P. officinarum* was examined at four

localities (Tab. 2). Not surprisingly, the highest within-population clonal diversity was found among sexual tetraploid hybrids. Regarding the apomictic hybrids, the pentaploids showed a higher clonal diversity within the populations than did the hexaploids (Tab. 2).

Discussion

Basic species
Pilosella bauhini

Our findings are in agreement with previously published data. The sexual tetraploids dominate in Slovakia and Hungary, whereas the apomictic pentaploids prevail in the Czech Republic and Germany (Rotreklová 2004; Rotreklová et al. 2005; Marhold et al. 2007; Mráz & Šingliarová 2009; Paule et al. 2017). The apomictic hexaploids were less frequently found in the Czech Republic and Germany and only rarely in Slovakia (Rotreklová 2004; Paule et al. 2017). Heptaploid *Pilosella bauhini* was rarely recorded in the Czech Republic (Krahulcová et al. 2009). The tetraploid plants from Slovakia, the morphology of which was considered in this paper, were classified as subspecies *magyarica*, whereas the pentaploid plants belonged to subspecies *bauhini*. This morphological distinction of two subspecies of *P. bauhini* that is correlated with ploidy, corresponds to conclusions of previous studies on this species in Central Europe (Rotreklová 2004; Rotreklová et al. 2005). A high within-population clonal diversity of the tetraploid *P. bauhini* (Tab. 2) corresponds to sexual nature of these plants in studied area.

Pilosella hoppeana subsp. *testimonialis*

The diploid ploidy level is in concordance with published data that originated from ten localities throughout Slovakia (Rotreklová et al. 2005; Marhold et al. 2007). The diploid ploidy level has exclusively been recorded also in those plants of *P. hoppeana* subsp. *testimonialis* that originated from other European countries, for example, from Germany (Paule et al. 2017) and Bulgaria (Mráz & Šingliarová 2009; Krahulcová et al. 2018). All plants of *P. hoppeana* subsp. *testimonialis* studied here are supposedly sexual, similarly to the other diploid species of the genus *Pilosella* (Krahulcová et al. 2000; Fehrer et al. 2007). The sexual breeding system for diploid *P. hoppeana* subsp. *testimonialis* was confirmed for plants originating, for example, from Bulgaria (Krahulcová et al. 2018).

Pilosella lactucella

Pilosella lactucella is exclusively diploid and sexual (for the reference data throughout Europe, see, e.g. Rotreklová et al. 2002; Suda et al. 2007). Thus, our present findings are in agreement with diploids that originated from other six localities in Slovakia (Marhold et al. 2007). A reduced pollen stainability up to 49 %, that was recorded in the sexual plant no. 917 from the Slovenský raj Mts. (Rotreklová & Krahulcová 2016), may suggest an influence of past hybridization. Otherwise, the diploid sexual species of *Pilosella* commonly show a high pollen stainability

(Rotreklová & Krahulcová 2016). On the other hand, the morphological characters of the plant no. 917 were very close to *P. lactucella*, and the DAPI flow cytometry did not distinguish the DNA content in this plant from the DNA content in a typical *P. lactucella* from the Krkonoše Mts., Czech Republic. The habitat of *P. lactucella* at the locality 7 (Gemerské Dechtáre village in Cerová vrchovina Highlands) is steppic grassland on loess with *Festuca valesiaca*, *Bothriochloa ischaemum* and different *Thymus* species, which differs from more common occurrence of this species on low productive wet grasslands. However, *P. lactucella* was given by Holub and Moravec from probably same habitats (pastures) that were situated rather close to locality 7 (Holub & Moravec 1965: 49).

Pilosella officinarum

Using both, the own and previously published data, Mráz et al. (2008) presented a generally contrasting pattern in the distribution of cytotypes of *P. officinarum* in Europe. The apomictic pentaploid *P. officinarum* is the most common cytotype in Slovakia, the hexaploids (mostly apomictic) are also rather common there, but the tetraploids are very rare (Mráz et al. 2008). So, the presence of pentaploids and hexaploids in the populations studied by us confirms these earlier findings (Mráz et al. 2008). The apomictic breeding system that we found in analysed plants, is also in agreement with previously published data (Mráz et al. 2008). The highest ploidy level found in the populations of *P. officinarum* so far, is the heptaploid one (reviewed by Mráz et al. 2008).

The monoclonal octoploid and apomictic progeny that were grown from seeds sampled at locality 6, had most likely originated via apomixis from an octoploid maternal plant(s). Although the isozyme phenotypes suggested monoclonality, two plants of this progeny had a strongly different paternal efficiency: whereas one plant produced a rather good pollen (pollen stainability exceeding 70%), the other plant produced no pollen grains at all (Rotreklová & Krahulcová 2016). The octoploid progeny of *Pilosella officinarum* that had originated in a cytotype-mixed population, suggests occurrence of an octoploid maternal parent, although it was not found at the locality. Such ploidy level would be a new highest ploidy level reported in a wild population of *P. officinarum*. The presence of pentaploids and hexaploids of *P. officinarum* at locality 6 (Tab. 2) is sufficient for a potential origin of octoploid cytotype there. Specifically, the octoploid plant(s) could originate (a) from hybridization of hexaploid and pentaploid plants combining unreduced and reduced gametes (6x + 2x or 5x + 3x) or (b) from hybridization between two pentaploid clones combining unreduced and reduced gametes (5x + 3x).

Although the both pentaploid and hexaploid cytotypes of *P. officinarum* (including highly probably also those plants originated from the locality 6) were facultatively apomictic, they surprisingly differed in the extent of within-population clonal diversity (Tab. 2). Namely, the hexaploid plants shared one common isozyme phenotype within each of the populations analysed, while the pentaploid clones comprised more isozyme phenotypes within a locality (Tab. 2). Such a different

within-population clonal diversity may reflect a significant effect of residual sexuality in the pentaploid cytotype, while the hexaploids may have a higher penetrance of apomictic reproduction that retains the maternal genotype. An alternative possibility is an independent colonization of the locality by several pentaploid clones of *P. officinarum* along with a single hexaploid clone.

Pilosella onegensis

Former publications usually included *P. onegensis* to *Pilosella caespitosa* (formerly *Hieracium caespitosum* Dumort.) and classified the diploids at subspecies level. For example, Sell and West (1976) present the current diploid species *P. onegensis* under the name of *Hieracium caespitosum* subsp. *brevipilum* (Nägeli et Peter) P. D. Sell. Skalińska & Kubień (1972) reported the diploid sexual plants from Poland under the name of *Hieracium pratense* Tausch (synonym of *H. caespitosum* Dumort.) subsp. *silvicolum* Zahn, that also corresponds to current species *P. onegensis*. Our finding of diploid *P. onegensis* in Slovenský raj Mts. is in agreement with other two published data from Slovakia that refer to Západné Tatry Mts. and Volovské vrchy Mts. (Marhold et al. 2007). The same diploid ploidy level has also been recorded in sexual plants from the Czech Republic (Krahulcová et al. 2001, the plants presented under the name of *Hieracium onegense* (Norrl.) Norrl.).

Intermediate (hybridogenous) species and hybrids
Pilosella glomerata
[*P. caespitosa – P. cymosa*]

The pentaploid plant of *P. glomerata* from Slovenský raj Mts. (plant no. 919) was included in previous study aimed on variation in the genome size in *Pilosella* (Suda et al. 2007). Both the tetraploid and the pentaploid cytotype of *P. glomerata* were reported so far from Germany (Paule et al. 2017), Czech Republic (Krahulec et al. 2004, 2008), and Scandinavia (Schuhwerk 2002). Our record of the hexaploid *P. glomerata* with chromosome number of 2n = 54 represents a new ploidy level for this hybridogenous species that is commonly apomictic (e.g. Krahulec et al. 2004, 2008).

Pilosella macrostolona
[*P. officinarum > P. caespitosa*]

The genome size in one of the hexaploid plants presented here (plant no. 927), was used in previous study aimed at another topic (Suda et al. 2007). Whereas an analogous hexaploid plant of *P. macrostolona* was also found in the Krkonoše Mts., Czech Republic (Krahulcová & Krahulec 1999), the tetraploid cytotype of this species was reported from Germany (Paule et al. 2017).

Tab. 2 Clonal structure in species-mixed populations of *Pilosella*.

Taxon	PL/RM	Locality label	No. of plants analysed	No. of isozyme phenotypes (clones)
P. bauhini	4*x*/sexual	6	2	2
		7	4	4
		8	4	4
		9	2	1
P. officinarum	5*x*/apomictic	6*	41	13
		7	3	2
		8	2	2
	6*x*/apomictic	6*	7	1
		10	8	1
		7 + 10	1 + 1	2
	8*x*/apomictic	6	7•	1
P. lactucella × *P. aurantiaca*	3*x*/apomictic	5	4	3
		2 + 5	1 + 4	3
P. bauhini × *P. officinarum*	4*x*/sexual	6	3	3
		7	3	2
		10	3	3
	5*x*/apomictic	7	4	3
		8	6	4
	6*x*/apomictic	8	3	1

The locality labels (order numbers) correspond to those given in Results. The differences in a combined pattern of four isozyme phenotypes (see Material and Methods) distinguished the individual clones (genotypes) among those plants that shared a common species/hybrid identity, ploidy level (PL) and reproduction mode (RM). The method used allowed to distinguish a minimum number of clones (genotypes) coexisting at the locality. * Reproduction mode not analysed: the apomictic reproduction of pentaploid and hexaploid *P. officinarum* in the population at locality 6 was inferred from apomictic nature of the other pentaploid/hexaploid plants of *P. officinarum* that originated from other localities. • The seven monoclonal octoploid apomictic plants were grown from seeds that were sampled from several fruiting plants (with undetermined ploidy level) at locality 6.

Pilosella schultesii
[*P. lactucella – P. officinarum*]

   The two tetraploid plants presented here (no. 923, 928) were used in previous study aimed at the variation in genome size in *Pilosella* (Suda et al. 2007). The pentaploid cytotype of *P. schultesii* has also been reported from Slovakia, namely, from the Nízke Tatry Mts. and Veľká Fatra Mts. (Marhold et al. 2007), and, additionally, also from Poland (Rotreklová et al. 2005). The triploid cytotype was found in Germany (Paule et al. 2017), whereas altogether three ploidy levels (3x, 4x and 5x) are known in *P. schultesii* from the Czech Republic (Krahulec et al. 2004, 2008; Rotreklová et al. 2005). The ploidy variation in this hybridogenous species

results likely from ploidy variation in one of the parents, *Pilosella officinarum*. Specifically, the triploid *P. schultesii* is most likely a hybrid between invariably diploid *P. lactucella* and the tetraploid *P. officinarum*, whereas both the tetraploid and the pentaploid cytotype of *P. schultesii* that were also recorded in Slovakia, resulted probably from hybridization of either the pentaploid or the hexaploid *P. officinarum* as the second parent. The both pentaploid and hexaploid *P. officinarum* is common in Slovakia (see above), and the tetraploid *P. schultesii* could therefore have originated via conjugation of a reduced gamete from *P. officinarum* (3n) and *P. lactucella* (n). Alternatively, an unreduced gamete from *P. lactucella* (2n) and a reduced gamete from *P. officinarum* (3n) probably gave rise to pentaploid *P. schultesii* in Slovakia.

Pilosella lactucella × P. onegensis

The hybrid plant (no. 924) co-occured at the locality 5 with both diploid putative parental species which differ from each other in the genome size (Suda et al. 2007). The inter-parental holoploid genome size that was detected in the diploid hybrid plant no. 924, really supported its putative origin (Suda et al. 2007). In addition, a similar plant with unknown ploidy level (the plant was not sampled for cultivation), the morphology of which resembled the hybridogenous species of *Pilosella floribunda* (Wimm. et Grab.) Fr. [the hybrid formula *P. caespitosa* ≥ *P. lactucella*], was recorded at the locality 2. So far, the diploid recent hybrid of *P. lactucella* and *P. onegensis* that is analogous to plant no. 924 from Slovenský raj Mts., has only been reported from the Krkonoše Mts., the Czech Republic (Krahulcová et al. 2001).

Pilosella lactucella × P. aurantiaca

This triploid hybrid from Slovenský raj Mts. was involved in previous study aimed at apomixis-linked markers in the populations of *Pilosella* (Hand et al. 2015). Although the triploid hybrid plants set abundantly the apomictic seeds, a low pollen stainability (up to 18%) that was recorded in two analysed plants no. 922 and 1539, may reflect the meiotic disturbances that are expectable during sporogenesis in a triploid plant (Rotreklová & Krahulcová 2016). Nevertheless, an autonomous apomixis in *Pilosella* does not require viable pollen for a successful development of apomictic seed (Bicknell & Koltunow 2004). The clonal diversity found in this hybrid at locality 5 (Tab. 2) may suggest an independent origin of three different clones from a repeated hybridization of parental species. The sharing of an identical isozyme phenotype between the plant no. 929 from locality 2 and one of the four plants from locality 5 (Tab. 2) may suggest a spread of this hybridogenous clone via apomictic seed from one locality to another. Whereas we sampled in the Slovenský raj Mts. the diploid *Pilosella lactucella* (see above), which is the first putative parent of the respective triploid hybrid, we did not found there the second parent, *P. aurantiaca*. But in fact, the parentage of *P. aurantiaca* is unquestionable on account of the orange-reddish colour of inflorescences in the hybrid. By all means, *Pilosella aurantiaca* was reported from several localities in the Slovenský raj Mts.

(Leskovjanská 2014), including just the both meadow areas (labeled as I6 Geravy and CH21 Za Stratenou in Leskovjanská 2014) from where originated the triploid hybrid of *P. lactucella* × *P. aurantiaca* (localities 2 and 5 in present study). The existing karyological data from Slovakia refer to pentaploid plants of *P. aurantiaca* from Belianske Tatry Mts. as well as from Západné Tatry Mts. (Marhold et al. 2007). So, the triploid hybrid *P. lactucella* × *P. aurantiaca* could have potentially originated from a simple cross of diploid (*P. lactucella*) and pentaploid (*P. aurantiaca*) parent via conjugation of reduced gametes.

The triploid hybrid from Slovenský raj Mts. is parallel to two hybridogenous species of the same putative origin, namely, *Pilosella fusca* (Vill.) Arv.-Touv. [*P. aurantiaca* > *P. lactucella*] and *Pilosella blyttiana* (Fr.) F. W. Schultz et Sch. Bip. [*P. aurantiaca* – *P. lactucella*] (Bräutigam 2017). The tetraploid *P. fusca* was found in the Krkonoše Mts., the Czech Republic (Krahulcová et al. 2013), similarly to the tetraploid apomictic *P. blyttiana* (Krahulcová et al. 2001). The ploidy level in the wild hybrids of *P. lactucella* and *P. aurantiaca* has also been reported from other countries in Central Europe, namely, from Poland (tetraploid and hexaploid hybrids, Skalińska 1967), Germany (triploid *P. blyttiana*, both the tetraploid and pentaploid *P. fusca*, Paule et al. 2017) and Austria (triploid *P. blyttiana*, Schuhwerk & Lippert 2002).

Pilosella bauhini – P. officinarum

We include here also the plants of this putative origin, the morphology of which adequately corresponds to intermediate species *P. brachiata* (DC.) F. W. Schultz & Sch. Bip. [*P. bauhini* < *P. officinarum*]. These plants were evidently recent hybrids and not stabilized hybridogenous species.

The hybrids *Pilosella bauhini* × *P. officinarum* co-occurred with their putative parental species at all six localities concerned, i.e. at localities 6, 7, 8, 9, 10 and 11. The variation in ploidy level in these hybrids (4x, 5x, 6x) corresponds to a potential contribution of parental cytotypes that were found in a respective area, and to combination of their reduced/unreduced gametes. So, the tetraploid *P. bauhini* could potentially hybridize via both reduced (2n) and unreduced (4n) gamete, while the second parent, the pentaploid/hexaploid *P. officinarum*, could exclusively hybridize via a reduced gamete (2n, 3n). The parental combination of a sexual (*P. bauhini*) and an apomictic (*P. officinarum*) parent gave rise to both sexual and apomictic hybrids. The pattern of clonal (genotype) variation among the tetraploid, pentaploid and hexaploid hybrid cytotypes (Tab. 2) may suggest that, similarly to *P. officinarum* (see above), the hybrid cytotypes possess a different level of (residual) sexuality that allows a repeated sexual mating. Another reason, not excluding the first one, may be that clonally diverse pentaploid hybrids originate more frequently than do the hexaploid hybrids.

The hybrids of *P. bauhini* and *P. officinarum* that have been examined karyologically so far in Central Europe, were substantially diverse in ploidy level. Some of these previously reported plants refer to intermediate species of the same parentage, to *Pilosella brachiata*. Specifically, the tetraploid and the pentaploid

Tab. 3 Summary of methods (represented by symbols A, B, C) that were used for the determination of reproduction mode in plants of *Pilosella* in this study. A – presence of good seed-set in open-pollinated versus emasculated capitula; B – screening of seed-progeny origins (FCSS method analysing the seed doublets); C – comparison between the maternal and progeny ploidy level(s), using those progenies that originated in mixed-ploidy populations. The last supporting approach involved (a) the FCSS analysis of pooled seed samples (six to ten seeds per sample) allowing to detect and quantify the ploidy of progeny embryos and/or (b) the FCM ploidy screening of the cultivated progeny plants that were grown from seeds. If the methods B and C were used, the number of analysed progeny individuals is given in parentheses. *Altogether seven monoclonal octoploid plants of *P. officinarum* were grown from seeds that were sampled in the field; out of them, four plants were analysed for reproduction mode. For the details see Material and Methods.

Taxon	Ploidy	Plant individual	Reproduction mode	Method used
P. bauhini subsp. *bauhini*	5*x*	1545A	apomictic	A, C (100)
	5*x*	1545B	apomictic	A
	5*x*	1546B	apomictic	A
P. bauhini subsp. *magyarica*	4*x*	1972	sexual	B (20)
	4*x*	1978	sexual	A
	4*x*	2081	sexual	A
	4*x*	1164	sexual	A
	4*x*	1941	sexual	A
	4*x*	1951	sexual	B (18)
	4*x*	1953	sexual	A
	4*x*	1955	sexual	B (20), C (3)
	4*x*	1925	sexual	A, B (6)
	4*x*	1926	sexual	B (20), C (17)
	4*x*	1937	sexual	B (20)
	4*x*	1940	sexual	B (20), C (69)
	4*x*	1966	sexual	A
P. lactucella	2*x*	1544	sexual	A, C (40)
	2*x*	1546A	sexual	A
	2*x*	917	sexual	A
P. officinarum	5*x*	1975	apomictic	A, B (16), C (9)
	5*x*	1944	apomictic	A

Tab. 3 - cont.

	5*x*	1945	apomictic	A
	5*x*	1954	apomictic	A
	5*x*	1927	apomictic	A, C (49)
	5*x*	1928	apomictic	A, C (64)
	5*x*	2026	apomictic	A
	6*x*	1542	apomictic	A
	6*x*	1548	apomictic	A
	6*x*	1551	apomictic	A
	6*x*	1952	apomictic	A
	6*x*	1960	apomictic	A
	6*x*	2023	apomictic	A
	8*x**	1977 PL1/4	apomictic	A, B (20), C (100)
	8*x**	1977 PL1/7	apomictic	A
	8*x**	1977 PL1/8	apomictic	C (89)
	8*x**	1977 PL1/11	apomictic	A
P. glomerata	6*x*	920	apomictic	A, C (90)
P. macrostolona	6*x*	927	apomictic	A, C (46)
	6*x*	918	apomictic	A, C (50)
P. schultesii	4*x*	930	apomictic	A
	5*x*	1943	apomictic	A
P. lactucella × onegensis	2*x*	924	sexual	A
P. lactucella × aurantiaca	3*x*	929	apomictic	A, C (40)
	3*x*	922	apomictic	A, C (50)
	3*x*	925	apomictic	A, C (60)
	3*x*	1538B	apomictic	A
	3*x*	1539	apomictic	C (200)
P. bauhini × officinarum	4*x*	1971	sexual	A
	4*x*	1973	sexual	A
	4*x*	1974	sexual	A
	4*x*	1947	sexual	A
	4*x*	1950	sexual	A, C (11)
	4*x*	1967	sexual	A
	4*x*	1961	sexual	A

Tab. 3 - cont.

	4*x*	1962/1	sexual	A, C (10)
	4*x*	1963	sexual	A
	4*x*	1964	sexual	A, B (19), C (11)
	4*x*	1962/2	apomictic	A
	4*x*	1962/3	apomictic	A
	5*x*	1547	apomictic	A
	5*x*	1550	apomictic	A
	5*x*	1946	apomictic	A
	5*x*	1948	apomictic	A
	5*x*	1948/2	apomictic	A
	5*x*	1956	apomictic	A, B (20), C (30)
	5*x*	1930	apomictic	A, B (4), C (7)
	5*x*	1931A	apomictic	A, C (50)
	5*x*	1933	apomictic	B (36), C (15)
	5*x*	1935	apomictic	A, C (3)
	5*x*	1936	apomictic	A
	5*x*	1969	apomictic	A
	5*x*	2010	apomictic	A
	5*x*	2012	apomictic	A
	5*x*	2019	apomictic	A
	6*x*	1929	apomictic	B (20), C (9)
	6*x*	1931B	apomictic	A
unclassified hybrid of *P. bauhini*	5*x*	1549	apomictic	A, C (98)
unclassified hybrid of *P. officinarum*	4*x*	1543	apomictic	A
hybrid of unknown origin	5*x*	921	apomictic	A

P. brachiata was recorded in two areas of Slovakia, Slovenský kras Mts. and Rožňavská kotlina Basin, respectively (Marhold et al. 2007). Altogether four ploidy levels (3x, 4x, 5x and 6x) were revealed in *P. brachiata* in Germany (Paule et al. 2017). The hybrids of *P. bauhini* and *P. officinarum* have also been studied in the hybridizing populations in the Czech Republic (Krahulcová et al. 2009, 2014; Křišťálová et al. 2010; Rosenbaumová & Krahulec 2015). These populations comprised an apomictic *P. bauhini* (usually 5x and/or 6x) and a sexual *P. officinarum* (4x), and altogether five

euploid hybrid cytotypes (4x, 5x, 6x, 7x and 8x) were recorded there, occasionally along with aneuploids. The hybrids with a high-ploidy level usually originated via fertilization of an unreduced female gamete from an apomictic parent (Krahulcová et al. 2009, 2014; Rosenbaumová & Krahulec 2015). Particular hybrid biotypes showed a diverse reproduction mode, comprising the sexual, apomictic (some of the high-polyploids with a high level of the residual sexuality), and less commonly also the sterile plants. In general, the hybrids originated from the Czech populations had a higher extent of ploidy variation (ranging from 4x to 8x, see above) than had the hybrids from Slovakia (ploidy variation ranging from 4x to 6x).

Acknowledgement

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