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ORIGINAL ARTICLE

Patterns of genetic variation in *Pilosella echioides* and its selected relatives: results of variation in ploidy level, facultative apomixis and past and present hybridization

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Abstract We used allozymes to elucidate the genetic variation of *Pilosella echioides* and *P. rothiana* in the Pannonian Basin and its relationship with morphology and modes of reproduction. The former species consists of sexual diploid, apomictic tetraploid, and very rare sexual tetraploid populations; the latter is exclusively tetraploid and apomictic. As expected, we detected the highest intrapopulation variation in diploid populations of *P. echioides*. Nonetheless, 73 % of populations of tetraploid *P. echioides* and 64 % of *P. rothiana* consisted of 2–7 multilocus allozyme genotypes, the means being 5.75 in *P. echioides* and 2.64 in *P. rothiana*. Both the proportion of distinguishable genotypes (*G/N*) per population and genotype diversity (*D*) per population significantly differed between diploid *P. echioides* (means 0.415 and 0.828, respectively) on the one hand and tetraploid *P. echioides* (means 0.252 and 0.387, respectively) and *P. rothiana* (means 0.213 and 0.347, respectively) on the other. Rather surprisingly, we found an excess of homozygotes (positive F_{IS}) in diploids, which indicates inbreeding. Tetraploids of *P. echioides* have most likely originated from only a few polyploidization events and have spread thanks to agamospermy—at least populations from the NW part of the area under study seem to be monophyletic. Genetic differences within the putatively hybridogeneous species *P. rothiana* are small. It seems plausible that it has a common origin and that it spreads independently of its parents (*P. echioides* and *P. officinarum*). A certain level of genetic diversity can be caused by residual sexuality or less likely by repeated polytopic hybridization between *P. echioides* and *P. officinarum*. *Pilosella sterrochaetia* is reported here from Hungary for the first time. It is an extremely rare primary diploid hybrid between diploid *P. echioides* and *P. leucopsilon*. Its intermediate nuclear genome size also confirms its hybrid origin.

Keywords *Pilosella* · Allozymes · Apomixis · Pannonian Basin · Genetic variation

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Introduction

The genetic structure of apomictic (agamospermous) populations has been a popular topic for the last three decades. Although a considerable amount of data has been gathered and several reviews of the topic have been published (e.g., Richards 1997; Hörandl et al. 2007), many questions still remain open. The genetic variation of apomicts apparently ranges from uniclonality to diversity comparable to that present in sexual populations (Gornall 1999). One unresolved issue is the origin of this genetic variation. Relationships between apomictic taxa/populations and their sexual ancestors may provide some answers. Genetic diversity in apomicts can generally be caused by (1) mutations, (2) backcrossing with sexual relatives, (3) meiotic recombination and cross-fertilization within

apomictic populations, and (4) multiple hybrid origins of apomicts from sexual ancestors (Gornall 1999).

The genus *Pilosella* Vaill. (sometimes treated as a subgenus of *Hieracium*) is among the most suitable groups for studies aimed at processes generating genetic variation in agamic complexes. It is one of the taxonomically most complicated genera of the temperate flora. Its diversity is caused by numerous factors, the most important being variation in breeding systems (facultative apomixis, allogamy, induced autogamy, haploid parthenogenesis, clonal growth), polyploidy and frequent hybridization (Krahulcová et al. 2000; Fehrer et al. 2007). Interactions among these factors have resulted in an extremely complicated pattern of morphological variation with several aspects that are typical of this genus, for example, striking local or regional differences in the diversity and morphology of hybrids and hybridogeneous types derived from the same parental species (e.g., Krahulec et al. 2008). One useful subject for studying these differences is *P. echioides* and its hybrid/hybridogeneous derivatives.

Pilosella echioides (Lumn.) F.W. Schultz & Sch. Bip. is a so-called 'basic' (nonhybridogeneous) perennial species with a large geographic area stretching from southern Russia westwards to central Europe (Bräutigam 1992). It grows in sandy and steppe grasslands, heathlands, sunny pine forests, and even on sand and rocks. Five different cytotypes (2*x*, 3*x*, 4*x*, 5*x*, 6*x*) have been reported (Trávníček et al. 2011). The distribution of individual cytotypes in the European part of the species' range shows a distinct geographic pattern with the highest ploidal diversity (up to five sexually reproducing auto-incompatible cytotypes on a small scale) growing in Southwestern Moravia (Czech Republic) and the adjacent part of Lower Austria (Trávníček et al. 2011). A contrasting pattern of cytotype distribution and modes of reproduction has been found in Hungary and southernmost Slovakia (for brevity hereafter referred to as the Pannonian Basin, though the area concerned is only a fraction of the whole region). Diploid and tetraploid populations are geographically separated from each other. No mixed-ploidy populations have been found in this region. The discovery of apomixis in tetraploid plants from Pannonia is the most surprising, as tetraploids in Austria and Czech Republic are sexual (Peckert et al. 2005; Trávníček et al. 2011). This raises many questions related to the role of sexual diploids and apomictic tetraploids in evolutionary processes. Perhaps most importantly, they may have participated in both past and recent hybridizations with other *Pilosella* species.

Besides *Pilosella echioides*, several of its derivatives, so-called 'intermediate species' (Nebenarten in German, i.e., species of presumably hybrid or hybridogeneous nature) have been reported from the Pannonian Basin (Zahn 1921–1923; Zahn 1922–1930; Soó 1970; Greuter 2006–2009). Some of them have been described from this region; others have their center of diversity here. It is reasonable to suppose that the high variability of *P. echioides* in the Pannonian Basin can account for the diversity of its intermediates. All this makes the Pannonian Basin a unique area with respect to the diversity of sect. *Echinina*, which is highly suitable for investigations of evolutionary processes and taxonomy.

Taxonomic evaluations of 'intermediate' *Pilosella* taxa often suffer from shortages of data about genetic and morphological variation, ploidy levels and modes of reproduction. These taxa can be (with a certain degree of simplification) divided into stabilized apomictic hybridogeneous taxa, which merit taxonomic classification, and primary hybrids originating repeatedly in mixed populations of parental taxa, which do not. In the Pannonian Basin, this applies first of all to taxa that are morphologically intermediate between *P. echioides* and *P. officinarum* Vaill. [*P. rothiana* (Wallr.) F. W. Schultz & Sch. Bip.—locally scattered in the area—and *P. bifurca* (M. Bieb.) F. W. Schultz & Sch. Bip.—rare] and between *P. echioides* and *P. leucopsilon* (Arv.-Touv.) Gottschl. [*P. sterrochaetia* (Nägeli & Peter) Soják—hitherto not reported and *P. erythrodonta* (Zahn) Soják—very rare]. It is possible to propose numerous scenarios of their evolutionary history, but hitherto available data are too scarce to draw even preliminary conclusions. First of all, there are no data on the ploidy levels and modes of reproduction of the intermediate species. Secondly, although *P. echioides* is without doubt one of the parents, it is difficult to decide, on the basis of morphology, which species is the second one (*P. leucopsilon* and *P. officinarum* are similar morphologically). To sum up, this species complex is a useful model group for reconstructing both past and recent hybridization processes.

Despite their drawbacks, allozymes are codominant markers, which makes them a useful tool for unravelling the origins of polyploids (Roose and Gottlieb 1976; Soltis and Rieseberg 1986; Soltis and Soltis 1989; Mahy et al. 2000; Rosquist and Prentice 2002; Crawford et al. 2006) or hybrids (Brochmann et al. 1992; Gauthier et al. 1998; Kaplan et al. 2002; Mráz et al. 2005). They can also be used to indirectly estimate breeding systems within populations (Kirschner et al. 1994; Sipes and Wolf 1997; Sydes and Peakall 1998). Allozymes are also useful for studying relationships at low systematic levels (Gottlieb 1984). Allozymes have successfully been used in the genus *Pilosella* to detect genetic diversity, often with respect to ploidy levels and reproductive modes or strategies (Krahulec et al. 2004; Kashin et al. 2005; Peckert et al. 2005; Tyler 2005; Bruun et al. 2007; Šingliarová et al. 2008, 2011; Krahulcová et al. 2009; Křišťálová et al. 2010). Flow cytometry has revealed a considerable difference in monoploid

genome sizes between *P. leucopsilon*/*P. officinarum* and *P. echioides* that facilitate the identification of their hybrids (Suda et al. 2007; in this paper, the respective parental species were referred to as *Hieracium hoppeanum* subsp. *testimoniale* Nägeli & Peter/*H. pilosella* L. and *H. echioides* Lumn., respectively).

The main goal of the present study was to investigate the pattern of genetic variation in diploid and tetraploid *P. echioides* (basic species) and in *P. rothiana* (intermediate species, *P. echioides* > *P. officinarum*), the degree of genetic divergence between *P. echioides* and *P. rothiana*, and the relationships between morphological and genetic variation and breeding systems (apomixis vs. sexuality) in the Pannonian Basin. The study provides sufficient primary data for reconstructing evolutionary processes in sect. *Echinina*, especially past and recent hybridization.

Materials and methods

Plants

We sampled living plants from four wild populations of diploid *P. echioides*, eleven populations of tetraploid *P. echioides* and eleven populations of *P. rothiana*. We selected the populations to representatively cover the distribution and morphological variation in the part of the Pannonian Basin under study (Fig. 1; Table 1). Sample size varied from 6 to 21 individuals per population (depending on population size). We transferred the plants sampled for cultivation to the Experimental garden of the Institute of Botany, Academy of Sciences of the Czech Republic in Průhonice. We ascertained the DNA ploidy level of all the plants using flow cytometry. We then examined the breeding systems of the same plants (all but five populations, see Table 1) and subjected them to allozyme analysis (all plants); selected plants were also used for chromosome counting (see below). In addition, we collected *P. echioides*, *P. leucopsilon* and their putative hybrids (*P. sterrochaetia*) at the locality LEI (nine plants in total). Putative hybrid plants are morphologically closer to *P. leucopsilon*. To test our hypothesis concerning the origin of the hybrids, we determined holoploid genome sizes (2*C* values; Greilhuber et al. 2007) of both hybrids and parental taxa. Voucher specimens are deposited in the herbarium of the Institute of Botany (PRA).

Flow cytometry

We prepared nuclei for both DNA ploidy level and genome size estimation following the now standard two-step procedure using Otto's buffers (Doležel et al. 2007). The DNA ploidy level was determined by a Partec CyFlow Space instrument equipped with a UV-diode chip, using *Bellis perennis* L. (2*C* = 3.38 pg; Schönswetter et al. 2007) as the internal reference standard and recording the fluorescence intensity of 3000 DAPI-stained particles (for methodological details, see Trávníček et al. 2011). Genome sizes were determined by a Partec CyFlow SL instrument (Partec GmbH, Münster, Germany) equipped with a 532 nm Samba solid-state laser (Cobolt AB, Solna, Sweden), using *Glycine max* (L.) Merr. 'Polanka' (2*C* = 2.50 pg; Doležel et al. 1994) as the internal reference standard and recording the fluorescence intensity of 5,000 particles stained with propidium iodide (for methodological details, see Suda et al. 2007). We only considered analyses whose coefficient of variation did not exceed 3 % for both the sample and the internal reference

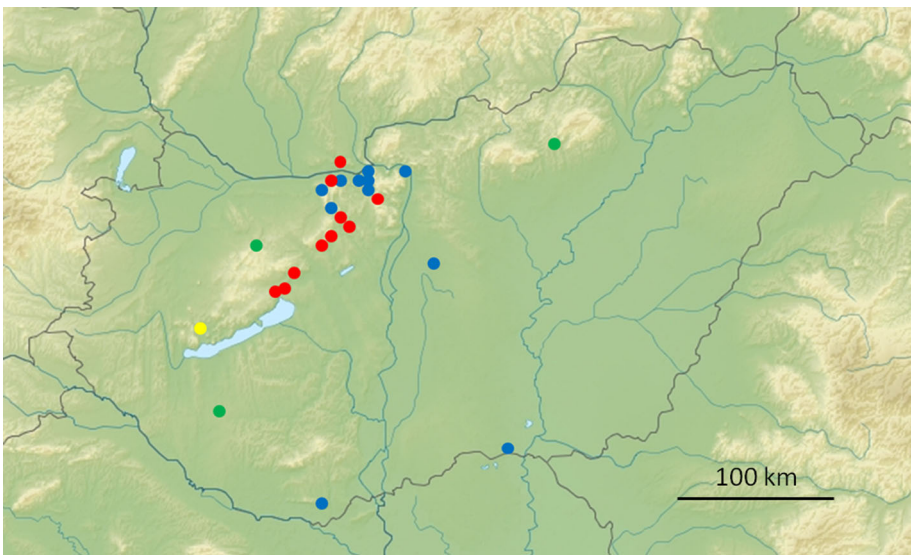

Fig. 1 Map of the area studied. *Green dots* 2*x Pilosella echioides*, *blue dots* 4*x P. echioides*, *red dots* *P. rothiana*, *yellow dot* common occurrence of 2*x P. echioides* and *P. rothiana*

Table 1 List of samples of *Pilosella echioides* (E) and *P. rothiana* (R)

Code	Species	Locality	Coordinates	Ploidy	MR
BOH	E	H, Somogy county: Nagybajom, Homokpuszta (near the road to Böhönye), sandy grassland, 3.5 km NW of the village, 151 m alt	46° 24′ 16″ N, 17° 28′ 34″ E	2*x*	S
FEN	E	H, Győr-Moson-Sopron county: Fenyőfő, sandy grassland 0.6 km SW of the village, 280 m alt	47° 20′ 42″ N, 17° 45′ 38″ E	2*x*	S
LEI	E	H, Veszprém county: Lesenceistvánd, grassland near the sand pit near the main road no 84, 125 m alt	46° 52′ 58″ N, 17° 21′ 35″ E	2*x*	S
SZK	E	H, Heves county: Szarvaskő, rocky slopes near a tourist path ca 1 km NW of the village, 340 m alt	47° 59′ 35″ N, 20° 19′ 48″ E	2*x*	S
ARA	E	H, Komárom-Esztergom county: Dorog, Arany-hegy, grassland on N slopes, 1.7 km SE of the church in the town, 170 m alt	47° 42′ 20″ N, 18° 44′ 22″ E	4*x*	A
HAR	E	H, Pest county: Újhartyán, disturbed grassland at the highway exit (Exit 44) NE of the village, 118 m alt	47° 13′ 29″ N, 19° 24′ 27″ E	4*x*	–
KEL	E	H, Bács-Kiskun county: Kelebia, pine forest margin on sand 5 km NW of the village, 130 m alt	46° 14′ 09″ N, 19° 38′ 21″ E	4*x*	S
LEA	E	H, Komárom-Esztergom county: Leányvár, NW grassy slopes of Kalap hegy, 1.6 km NW of the church in the village (Vaskapupuszta), 150 m alt	47° 41′ 40″ N, 18° 45′ 35″ E	4*x*	–
NYE	E	H, Komárom-Esztergom county: Nyergesújfalu, SW margin of the village (settlement between Nyergesújfalu and Labatlan), 155 m alt	47° 44′ 55″ N, 18° 31′ 28″ E	4*x*	–
SAT	E	H, Komárom-Esztergom county: Dorog, Sátor-kő, top of the hill, 1.6 km ENE of the church in the town, rocks and grasslands, 165 m alt	47° 43′ 13″ N, 18° 45′ 05″ E	4*x*	A
SZO	E	H, Komárom-Esztergom county: Szomód, Les-hegy hill, around the top, 2.2 km NW of the village, 230 m alt	47° 41′ 56″ N, 18° 19′ 24″ E	4*x*	A
TAT	E	H, Komárom-Esztergom county: Tatabánya-Kertváros, vast places between the Rakocsi Ferenc út and Szvatopluk út, 145 m alt	47° 34′ 49″ N, 18° 22′ 24″ E	4*x*	–
TOK	E	H, Komárom-Esztergom county: Tokod-altáró, Altáróbanyatelep, sand pit (upper part) S of the village, 188 m alt	47° 43′ 23″ N, 18° 41′ 42″ E	4*x*	A
TOT	E	H, Pest county, Szentendrei sziget: Tótfalu, dry grassland on sand dunes 4.3 km NNW of the village center, 103 m alt	47° 47′ 30″ N, 19° 04′ 42″ E	4*x*	A
VIL	E	H, Baranya county: Villány, above the quarry between Villány and Nagyharsány (Szoborpark), stony slope, 190 m alt	45° 51′ 25″ N, 18° 25′ 45″ E	4*x*	S
BAG	R	H, Vértes Mts., Fejér county: Csákvár, S slopes of Bagó-hegy, near a carriageway, 2.2 km NW of the church in the village, 203 m alt	47° 24′ 38″ N, 18° 26′ 48″ E	4*x*	A
BOD	R	H, Vértes Mts., Fejér county: Bodmér, dry grassland in front of the cemetery, 205 m alt	47° 27′ 01″ N, 18° 31′ 43″ E	4*x*	A
CSA	R	H, Vértes Mts., Fejér county: Gánt, old quarry near the local road to Csákvár, 2 km NE of the church in the village, 265 m alt	47° 23′ 55″ N, 18° 24′ 38″ E	4*x*	A
HAJ	R	H, Veszprém county: Hajmáskér, dry grasslands 2.5 km SW of the village center, 187 m alt	47° 07′ 43″ N, 18° 00′ 15″ E	4*x*	A
JUR	R	Sk, Nitriansky kraj, distr. Nové Zámky: Jurský Chlm, near the road 0.5 km S of the village, 110 m alt	47° 47′ 59″ N, 18° 32′ 08″ E	4*x*	A
KAD	R	H, Veszprém county: Kádárta (near Veszprém), ca 2.2 km SE of the village center, 248 m alt	47° 06′ 16″ N, 17° 57′ 49″ E	4*x*	A
LES	R	H, Veszprém county: Lesenceistvánd, grassland near the sand pit near the main road no 84, 125 m alt	46° 52′ 58″ N, 17° 21′ 35″ E	4*x*	–
PIL	R	H, Pest county, Budai hegység: Pilisszentiván, SE slopes of Mt. Kakukk-hegy, 264 m alt	47° 37′ 02″ N, 18° 52′ 15″ E	4*x*	A
SUT	R	H, Komárom-Esztergom county: Süttő, near a sand pit 2.9 km WSW of the village, 121 m alt	47° 44′ 38″ N, 18° 24′ 31″ E	4*x*	A
SZR	R	H, Komárom-Esztergom county: Szár, slopes above parking place at the road no 1 (direction Tatabánya), 1,2 km NE of the village, 220 m alt	47° 29′ 21″ N, 18° 31′ 32″ E	4*x*	A
VAR	R	H, Veszprém county: Várpalota, near the road to Bakonycsernye, 2.8 km NNE of the town center, 248 m alt	47° 13′ 24″ N, 18° 08′ 30″ E	4*x*	A

MR mode of reproduction (A, apomictic; S, sexual; –, not determined), *H* Hungary, *Sk* Slovakia

standard. To estimate genome size, we performed three runs of the analysis per plant (each analysis on a different day); the differences among these three estimates of genome size in an individual plant did not exceed 1.7 %.

Chromosome numbers and modes of reproduction

Chromosomes of *Pilosella sterrochaetia* (2 plants, locality LEI), selected plants of *P. echioides* (2 LEI, 2 SAT) and *P. rothiana* (2 HAJ, to confirm the chromosome numbers in plants also used for cytometric analyses) and *P. leucopsilon* (putative parental species of *P. sterrochaetia*, 2 LEI) were counted in root-tip meristems pretreated in a solution of saturated *p*-dichlorobenzene and stained by lacto-propionic orcein as described by Krahulcová and Krahulec (1999).

We determined the mode of reproduction following the procedure used, for example, by Gadella (1987) or Krahulcová and Krahulec (1999). We treated diploid and tetraploid plants differently. In diploid plants (populations BOH, FEN, LEI and SZK), we (1) bagged the inflorescences of five plants per population in nylon mesh to prevent pollination, and (2) left the other inflorescences of the same plants uncovered to allow open pollination; the plants were grown together, and most of them flowered simultaneously. In tetraploids (in which we expected apomixis based on previous studies, see Table 1), we (1) emasculated 3–5 capitula per plant (2–5 plants per population) by cutting off the upper part of each capitulum before anthesis; after flowering, we isolated the capitula using nylon mesh bags to prevent loss of ripe seeds, and (2) allowed the capitula to be open-pollinated. For each flower head, we determined the number of developed (full) and empty seeds, and calculated the proportion of developed (full) seeds. In the emasculation experiments, the proportion of developed (full) seeds was either consistently higher than 75 % (such plants are taken as apomicts) or zero (such plants were further tested under open pollination conditions for sexuality or sterility).

Electrophoresis

Enzymes were extracted from young leaves of cultivated plants. Approximately 1 cm^2 (cca 40 mg) of fresh leaf tissue was ground in ice-cold extraction buffer following Kato (1987): 0.1 M Tris–HCl (pH = 8.0), 70 mM mercaptoethanol, 26 mM sodium metabisulphite, 11 mM L-ascorbic acid, 4 % (w/v) soluble PVP-40 and Dowex Cl resin (1–8X); the pH of the buffer was adjusted by adding the ascorbate. Crude homogenates were centrifuged at 15,000 rpm for 10 min. Supernatants were stored at −75 °C. Electrophoresis (PAGE) was carried out using a 8.16 % separation polyacrylamide gel with a 1.82 M Tris–HCl buffer (pH 8.9), 4 % stacking gel buffer (0.069 M Tris–HCl, pH 6.9) and electrode buffer (0.02 M Tris, 0.24 glycine, pH 8.3). PAGE was performed for seven isozyme systems: shikimate dehydrogenase SHDH (EC 1.1.1.25), 6-phosphogluconate dehydrogenase 6-PGDH (EC 1.1.1.44), superoxide dismutase SOD (EC 1.15.1.1), leucine aminopeptidase LAP (EC 3.4.11.1), dihydrolipoyl dehydrogenase DIA (EC 1.8.1.4), alcohol dehydrogenase ADH (EC 1.1.1.1), and aspartate aminotransferase AAT (EC 2.6.1.1). The systems DIA and AAT exhibited two and three zones of activity, respectively, interpreted as two and three putative loci (the fastest-moving locus was denoted by the number '1'). Alleles were denoted sequentially with the fastest one coded as 'a'.

Data analysis

To provide a measure of the level of genetic variation within populations, we calculated the following statistics: P proportion of polymorphic loci, ΣA sum of alleles, A mean number of alleles per locus, H_o observed heterozygosity, and H_e expected heterozygosity for diploids (following Nei 1973). In the case of diploids, we calculated these parameters with the help of Popgene (Yeh et al. 1999) and Fstat (Goudet 1995); in the case of polyploids, we calculated them by hand. We estimated genotypic variation using standard parameters for clonal populations (Ellstrand and Roose 1987; Eckert and Barrett 1993): number of genotypes (G), proportion of distinguishable genotypes (i.e., the number of genotypes to the number of samples, G/N), and number of unique genotypes (G_{uni}). We calculated multilocus genotype diversity D (modified Simpson diversity index) as $D = 1 - \Sigma n_i(n_i - 1)/N(N - 1)$, where n_i is the number of individuals of genotype i (Pielou 1969; but see also Noyes and Soltis 1996; Hörandl et al. 2000; Liston et al. 2003). The D value ranges from 0 to 1, 0 meaning that all individuals represent the same multilocus genotype and 1 meaning that each individual has a unique multilocus genotype. We determined all parameters for each population and each of the three groups (diploid *P. echioides*, tetraploid *P. echioides* and *P. rothiana*). For each diploid population, we calculated F_{IS} and Weir and Cockerham's estimation of Φ or F_{ST} (Fstat, Goudet 1995). We then tested for differences in selected genetic parameters among diploid *P. echioides*, tetraploid *P. echioides* and *P. rothiana* by an ANOVA with an Unequal N HSD post hoc comparison test. We compared the values between pairs of taxa using t tests. Some of the genetic parameters violated the assumption of normality (assessed by a Shapiro–Wilk test), so we tested them using nonparametric Kruskal–Wallis sum rank tests and multiple comparisons of groups.

We performed hierarchical clustering (UPGMA) to gain insight into the genetic relationships within the complete dataset. We used Tomiuk–Loeschke distances (Tomiuk

et al. 1998), which are useful for datasets with both diploid and polyploid populations, calculated using POPDIST version 1.2.4 (Tomiuk et al. 2009). For clustering, we used SYN-TAX 2000 (Podani 2001). We inferred the correlation between pairwise Tomiuk–Loesche genetic distances and geographical distances among populations of diploid *P. echioides* using Mantel's test with 10,000 random permutations as implemented in IBDWS version 3.23 (Jensen et al. 2005).

Results

DNA ploidy levels and modes of reproduction in *Pilosella echioides* and *P. rothiana*

We detected two ploidy levels, diploid and tetraploid, in *Pilosella echioides*. While diploids proved to be sexual and allogamous, all but two (KEL, VIL) of the tetraploid populations examined were apomictic. By contrast, *P. rothiana* was uniform in both its ploidy ($4x$) and mode of reproduction (apomixis) (Table 1). We found the chromosome number of $2n = 2x = 18$ in *P. echioides* from LEI and the chromosome number of $2n = 4x = 36$ in *P. echioides* from SAT and *P. rothiana* from HAJ.

Allozyme diversity

Allozyme analysis revealed ten loci representing seven enzyme systems. Despite the generally complicated genetic interpretation in polyploids (Gottlieb 1981; Weeden and Wendel 1989; Obbard et al. 2006), the resolution of gels and staining intensity enable us to take relative band intensity into account. We compared bands of polyploids with banding patterns of related diploids and interpreted differences in banding intensity as different allelic dosages (see, e.g., Arft and Ranker 1998; Hörandl et al. 2000; Hardy and Vekemans 2001; Šingliarová et al. 2011).

Ten loci yielded a total of 38 alleles (Table 2). Altogether eighteen alleles were detected in *P. echioides* (both cytotypes) and *P. rothiana*. Diploid and tetraploid cytotypes of *P. echioides* shared a total of eighteen alleles. Twelve alleles detected in tetraploids were not present in diploids, and two alleles of diploids were absent in tetraploids. However, the frequency of these unique alleles was, as a rule, extremely low (Table 2). *Pilosella rothiana* exhibited five specific alleles (*Lap-b*, *6Pgdh1-c*, *6Pgdh1-e*, *Aat3-a* and *Aat3-e*), but some of them were very rare, often found in only a single population (*Lap-b*, *6Pgdh1-e* and *Aat3-e*). The most valuable for proper delimitation of *P. rothiana* seems to be allele *Aat3-a* (detected in all plants of *P. rothiana*); allelle *6Pgdh1-c* was completely absent in *P. echioides*, but very unevenly distributed and only moderately frequent in *P. rothiana*. Conversely, allele *Aat2-c* was completely absent in *P. rothiana*, but its frequency in *P. echioides* was extremely low and distribution among populations uneven. *Pilosella rothiana* shared five alleles with tetraploid *P. echioides*, being absent in diploid *P. echioides*. However, some of them were extremely rare in tetraploid *P. echioides*.

Cluster analysis (UPGMA) clearly separated *P. echioides* and *P. rothiana* (Fig. 2). Within the *P. echioides* cluster, a group of three (of four) diploid populations (BOH, FEN, LEI) can be recognized, as well as a group of three populations where some introgression from *P. piloselloides* cannot be fully excluded (LEA, SZO, TAT). The Mantel test performed for three datasets (diploid *P. echioides*, tetraploid *P. echioides* and *P. rothiana*) showed that (log)geographic distances are not correlated with genetic distances among populations (diploid *P. echioides*—$r = 0.4836$, $p = 0.2836$; tetraploid *P. echioides*—$r = 0.2345$, $p = 0.1293$; and *P. rothiana*—$r = -0.0230$, $p = 0.5007$).

Genetic diversity values are given in Table 3 (population level) and Table 4 (species/cytotypes level). The groups do not significantly differ in the number of alleles per locus ($F_{3,23} = 2.757$, $p = 0.085$) and the percentage of polymorphic loci ($F_{3,23} = 0.044$, $p = 0.957$). They do, however, markedly differ from each other in observed heterozygosity ($F_{3,23} = 62.67$, $p < 0.001$), the highest being in *P. rothiana* and the lowest in diploid *P. echioides*. The number of multilocus genotypes per population varies from two to seven (mean 5.75) in diploid *P. echioides*, from one to five (mean 2.64) in tetraploid *P. echioides* and from one to seven (mean 2.55) in *P. rothiana*, respectively. The proportion of distinguishable genotypes (*G/N*) per population ranges from 0.267 to 0.533 (mean 0.415) in diploid *P. echioides*, from 0.063 to 0.571 (mean 0.252) in tetraploid *P. echioides* and from 0.067 to 0.467 (mean 0.213) in *P. rothiana*. Diploid *P. echioides* and *P. rothiana* significantly differ in *G/N* values ($t = 3.058$, $d.f. = 13$, $p = 0.009$). Most genotypes are population-specific (total of 28 genotypes in tetraploid *P. echioides*, only two of them observed in more than one population; total of 23 genotypes in *P. rothiana*, only five of them observed in more than one population) and species-specific (only one genotype shared by both species; the explanation is unclear). The three groups do not differ in the number of unique genotypes per population ($F_{3,23} = 0.441$, $p = 0.649$). The frequency of particular genotypes within apomictic populations is usually uneven, with one predominating and two (or more) minor genotypes. Genotype diversity (D) per population varies from 0.743 to 0.886 (mean 0.828) in diploid *P. echioides*, from 0 to 0.810 (mean 0.387) in tetraploid *P. echioides* and from 0 to 0.868 (mean 0.347) in *P. rothiana*; D of diploids differs from that of tetraploid *P. echioides* ($p = 0.034$) and *P. rothiana* ($p = 0.017$), respectively. Populations of

Table 2 Allelic frequencies in 10 loci in all analysed populations

	P. echioides 2*x*				*P. echioides* 4*x*										
	FEN	SZK	BOH	LEI	ARA	HAR	KEL	LEA	NYE	SAT	SZO	TAT	TOK	TOT	VIL
N	14	15	15	14	12	6	6	15	15	16	15	13	21	9	7
PL	2*x*	2*x*	2*x*	2*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*
Lap-a	0	0	0	0	0	0	0	0	0	0	0.1	0.04	0	0	0
Lap-b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lap-c	0	0	0	0	0	0	0	0	0.7	0	0	0	0	0	0
Lap-d	1	1	0.87	1	1	0.17	1	1	0.3	0.75	0.9	0.96	1	1	1
Lap-e	0	0	0	0	0	0	0	0	0	0.25	0	0	0	0	0
Lap-f	0	0	0.13	0	0	0.83	0	0	0	0	0	0	0	0	0
6Pgdh-1-a	0.79	0.4	0.2	1	0.25	0.71	0.25	0.51	0.48	0.75	0.62	0.42	0.49	0.89	0.64
6Pgdh-1-b	0.21	0.6	0.8	0	0.75	0.29	0.75	0.02	0.52	0.25	0.38	0.56	0.5	0.11	0.36
6Pgdh-1-c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6Pgdh-1-d	0	0	0	0	0	0	0	0.47	0	0	0	0.02	0.01	0	0
6Pgdh-1-e	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Shdh-a	0	0	0	0	0	0	0	0	0	0	0.05	0.04	0	0	0
Shdh-b	0	0	0	0	0	0	0.5	0	0.5	0	0.05	0.19	0.37	0	0
Shdh-c	1	1	1	1	1	1	0.5	1	0.5	1	0.68	0.77	0.63	1	1
Shdh-d	0	0	0	0	0	0	0	0	0	0	0.22	0	0	0	0
Dia-1-a	0	0	0	0	0	0	0	0	0	0	0.05	0.02	0	0	0.07
Dia-1-b	1	1	1	1	1	0.96	0.75	1	0.5	1	0.92	0.75	0.99	0.78	0.93
Dia-1-c	0	0	0	0	0	0.04	0.25	0	0.5	0	0.03	0.19	0	0.16	0
Dia-1-d	0	0	0	0	0	0	0	0	0	0	0	0.04	0.01	0.06	0
Dia-2-a	0	0	0	0	0	0.42	0	0.25	0	0	0.42	0.13	0	0.33	0
Dia-2-b	1	1	1	0.89	1	0.58	1	0.75	1	1	0.58	0.87	1	0.67	1
Dia-2-c	0	0	0	0.11	0	0	0	0	0	0	0	0	0	0	0
Sodx-a	1	1	1	1	1	1	1	1	1	1	0.95	0.98	1	1	1
Sodx-b	0	0	0	0	0	0	0	0	0	0	0.05	0.02	0	0	0
Adhx-a	0	0	0	0	0	0	0	0	0.23	0	0	0	0	0	0
Adhx-b	0.96	1	1	1	0.75	0.96	1	0.98	0.77	0.75	0.92	0.85	0.99	1	0.86
Adhx-c	0.04	0	0	0	0.25	0.04	0	0.02	0	0.25	0.08	0.15	0.01	0	0.14
Aat-1-a	0	0.13	0	0	0	0	0	0	0	0	0	0	0	0	0
Aat-1-b	0.68	0.87	0.67	0.54	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	0.39	0.36
Aat-1-c	0.07	0	0.13	0.21	0	0	0	0.5	0	0.5	0.5	0	0	0.22	0
Aat-1-d	0.25	0	0.2	0.25	0.5	0.5	0.5	0	0.75	0	0	0.5	0.5	0.39	0.64
Aat-2-a	0.25	0.13	0.2	0.18	0	0.42	0.5	0	0.23	0	0	0.19	0	0	0
Aat-2-b	0.43	0.87	0.47	0.56	0.5	0.16	0	1	0.07	1	1	0.62	0.54	0.78	1
Aat-2-c	**0.07**	0	**0.13**	**0.1**	0	0	0	0	**0.7**	0	0	0	0	**0.22**	0
Aat-2-d	0.25	0	0.2	0.16	0.5	0.42	0.5	0	0	0	0	0.19	0.46	0	0
Aat-3-a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aat-3-c	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Aat-3-e	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	P. rothiana 4x										
	BAG	BOD	CSA	HAJ	JUR	KAD	LES	PIL	SUT	SZR	VAR
N	7	15	15	15	17	17	7	7	11	8	8
PL	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*	4*x*
Lap-a	0	0.3	0	0	0	0.35	0	0.21	0	0.5	0.5
Lap-b	0	**0.05**	0	0	0	0	0	0	0	0	0

Table 2 continued

	P. rothiana 4x										
	BAG	BOD	CSA	HAJ	JUR	KAD	LES	PIL	SUT	SZR	VAR
Lap-c	0	0	0	0	0	0	0	0	0	0	0
Lap-d	1	0.65	1	0.75	1	0.65	1	0.79	1	0.5	0.5
Lap-e	0	0	0	0	0	0	0	0	0	0	0
Lap-f	0	0	0	0.25	0	0	0	0	0	0	0
6Pgdh-1-a	0.75	0.6	0.65	0.73	0.5	0.57	0.75	0.36	0.66	0.75	0.75
6Pgdh-1-b	0	0.29	0.17	0	0.5	0.18	0	0.64	0	0.25	0.25
6Pgdh-1-c	**0.25**	**0.08**	**0.13**	0	0	**0.04**	**0.25**	0	0	0	0
6Pgdh-1-d	0	0	0.03	0.27	0	0.21	0	0	0.44	0	0
6Pgdh-1-e	0	**0.03**	**0.02**	0	0	0	0	0	0	0	0
Shdh-a	0.5	0.33	0.25	0.25	0.44	0.31	0.5	0.5	0.5	0.28	0.28
Shdh-b	0	0.17	0.25	0.25	0.06	0.06	0	0	0	0	0
Shdh-c	0.5	0.5	0.5	0.5	0.5	0.63	0.5	0.5	0.5	0.72	0.72
Shdh-d	0	0	0	0	0	0	0	0	0	0	0
Dia-1-a	0.25	0.25	0.25	0.25	0.26	0.35	0.25	0.25	0.25	0.25	0.25
Dia-1-b	0.75	0.75	0.5	0.5	0.71	0.58	0.75	0.75	0.75	0.75	0.75
Dia-1-c	0	0	0.25	0.25	0.03	0.07	0	0	0	0	0
Dia-1-d	0	0	0	0	0	0	0	0	0	0	0
Dia-2-a	0	0.05	0	0	0	0	0	0	0	0	0
Dia-2-b	1	0.95	1	1	1	1	1	1	1	1	1
Dia-2-c	0	0	0	0	0	0	0	0	0	0	0
Sodx-a	0.75	0.75	0.75	0.77	0.78	0.75	0.75	0.75	0.75	0.75	0.75
Sodx-b	0.25	0.25	0.25	0.23	0.22	0.25	0.25	0.25	0.25	0.25	0.25
Adhx-a	0	0	0	0	0	0	0	0	0	0	0
Adhx-b	0.75	0.75	0.5	0.5	0.78	0.66	0.75	0.89	0.75	0.75	0.75
Adhx-c	0.25	0.25	0.5	0.5	0.22	0.34	0.25	0.11	0.25	0.25	0.25
Aat-1-a	0	0	0.25	0.25	0	0.07	0	0	0	0	0
Aat-1-b	0.25	0.72	0.75	0.75	0.25	0.93	0.5	1	0.5	1	1
Aat-1-c	0	0	0	0	0.75	0	0	0	0	0	0
Aat-1-d	0.75	0.28	0	0	0	0	0.5	0	0.5	0	0
Aat-2-a	0	0	0.25	0.25	0	0.07	0	0	0.5	0	0
Aat-2-b	1	1	0.75	0.75	1	0.93	1	1	0	1	1
Aat-2-c	0	0	0	0	0	0	0	0	0	0	0
Aat-2-d	0	0	0	0	0	0	0	0	0.5	0	0
Aat-3-a	**0.25**	**0.25**	**0.25**	**0.25**	**0.22**	**0.07**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**
Aat-3-c	0.75	0.75	0.75	0.75	0.78	0.75	0.75	0.75	0.75	0.75	0.75
Aat-3-e	0	0	0	0	0	**0.18**	0	0	0	0	0

Alleles unique to species are indicated in bold. Alleles *Aat3-b* and *Aat3-d* are intentionally lacking because they were detected in plants outside the area studied

PL ploidy level, *N* sample size

diploids differ from each other with respect to F_{IS}. Excess homozygotes (positive F_{IS}), indicating a certain level of inbreeding, were detected in BOH, FEN, and LEI; excess heterozygotes were found in SZK (Table 3). The value Φ (F_{ST}) = 0.171 indicates among-population differentiation, as might be supposed from the geographic distances.

Diploid hybrids

We found putative hybrid plants (*Pilosella sterrochaetia*) in a mixed population of *P. echioides* ($2n = 2x = 18$, population LEI in this study) and *P. leucopsilon* ($2n = 2x = 18$); all of them proved to be diploid

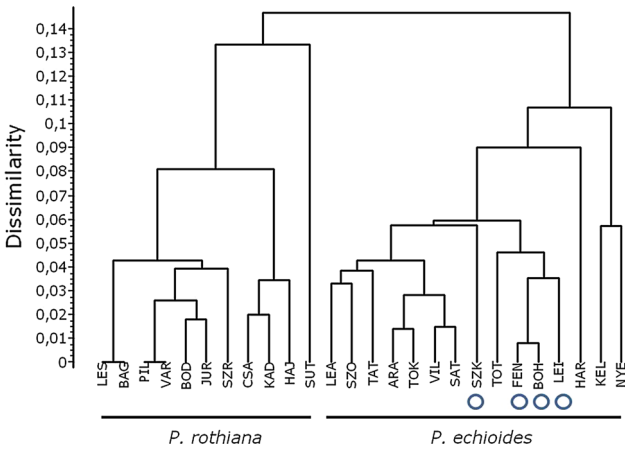

Fig. 2 Cluster analysis (UPGMA) of the populations studied. Diploid populations are marked by *circles*

($2n = 2x = 18$). The detected variation in holoploid genome size supports their assumed hybrid origin. The difference between the C values of the two parental species was approximately 20 %, and the mean C value of the putative hybrids was half-way between the values of their putative parents (Table 5). The hybrids set some seeds after open pollination (though the seed set was not quantified exactly) in the experimental garden, where numerous cultivated *Pilosella* taxa/cytotypes could serve as pollen donors. Capitula of different hybrid individuals contained variable (but generally low) amounts of viable seeds.

Discussion

Genetic variation and modes of reproduction

In the present paper, we confirm ploidal differentiation of *P. echioides* in the Pannonian Basin (Trávníček et al. 2011). Agamospermy in Pannonian tetraploids has also been reported (Peckert et al. 2005). Our discovery of tetraploid sexuals (KEL, VIL) in southern Hungary is rather surprising and sheds new light on the overall picture of ploidal and reproductive variation in *P. echioides*. Tetraploid sexuals had previously been known only from the Czech Republic and the eastern part of Austria (Trávníček et al. 2011). By contrast, *P. rothiana* is considerably more uniform with respect to its chromosome number/ploidy level and reproduction mode—all but one of the chromosome counts/ploidy levels detected by flow cytometry (triploid, southern Moravia, Rotreklová in Rotreklová et al. 2005) refer to $2n = 36$ chromosomes/the tetraploid level (plants from Austria, the Czech Republic and Germany; Schuhwerk and Lippert 1997, 2002; Rotreklová et al. 2002; Křišťálová et al. 2010). All hitherto analysed plants were apomictic (Rotreklová et al. 2002, 2005; Křišťálová et al. 2010).

The chromosome number and genome size of *P. sterrochaetia* are reported here for the first time. Based on these indices and their morphological intermediacy (although the plants are more similar to *P. leucopsilon*), the plants are most likely primary hybrids between *P. echioides* and *P. leucopsilon*. Besides a limited capability for generative reproduction, they can spread by long aboveground stolons. No plants that would morphologically resemble backcrossed individuals were observed at the locality.

Rather surprising is the excess of homozygotes (positive F_{IS}), which indicates inbreeding in all diploid sexual allogamous populations except SZK. In the case of the populations LEI and BOH, it might be explained by population history and size—both populations are small, at sites recently influenced by man and perhaps rather new. The species has nevertheless been collected repeatedly and is clearly established in the respective regions. It is more difficult to plausibly explain the excess homozygotes in the FEN population because this population, which grows in a sandy steppe grassland, is large and not fragmented. However, the extent of suitable habitats naturally fluctuated in the past, and repeated bottle necks are probable. On the other hand, population SZK occupies more or less stable habitats, probably with a continuously treeless history (rocky outcrops), which well coincides with its genetic diversity. We are aware that the limited number of diploid populations (they are most probably very rare in Hungary at all) analysed does not allow us to draw a final conclusion about their genetic diversity.

Furthermore, we should interpret our data in the context of the vegetation history of the Pannonian Basin. *Pilosella echioides* probably occupied a cold glacial Pannonian steppe and survived the Holocene in a deciduous wooded steppe, where varied canopy composition most likely facilitated the persistence of heliophilous species (Magyari et al. 2010). Anthropogenic activities since the Neolithic period in all likelihood caused the spread of *P. echioides* into new open habitats, yet populations probably fluctuated in both time and space. The historical distribution and abundance of diploid populations cannot be traced, so we cannot determine the extent of their retreat. In contrast to our populations, excess heterozygotes (negative F_{IS} values) and higher values of H_o (0.270 and 0.175, respectively, depending on the region; 0.121 in diploid *P. echioides*) were detected in high-mountain diploid populations of *Pilosella ullepitschii* from the Carpathians (Šingliarová et al. 2008) and *P. rhodopea* (0.255) from Bulgaria and Romania (Šingliarová et al. 2011).

Genetic variation in tetraploid *P. echioides* and *P. rothiana* is relatively high and contradicts the supposedly prevailing apomictic mode of reproduction at first glance. Inter-population differentiation accounts for most

Table 3 Summary of genetic variation for 10 loci in a total of 26 populations of diploid and tetraploid *Pilosella echioides* and *P. rothiana*

Code	PL	*N*	*P*	H_o	H_e	$\sum A$	*A*	F_{IS}	*G*	*G/N*	G_{uni}	*D*
P. echioides 2*x*												
FEN	2*x*	14	0.4	0.136	0.156	17	1.7	0.168	6	0.429	1	0.835
SZK	2*x*	15	0.3	0.120	0.094	13	1.3	−0.241	4	0.267	1	0.743
BOH	2*x*	15	0.4	0.120	0.173	17	1.7	0.339	7	0.533	4	0.886
LEI	2*x*	14	0.3	0.107	0.148	16	1.6	0.311	6	0.429	3	0.846
P. echioides 4*x*												
ARA	4*x*	12	0	0.4	–	14	1.4	–	1	0.083	1	0
HAR	4*x*	6	0.5	0.42	–	18	1.8	–	3	0.5	3	0.733
KEL	4*x*	6	0	0.5	–	15	1.5	–	1	0.167	0	0
LEA	4*x*	15	0.2	0.31	–	15	1.5	–	2	0.133	2	0.133
NYE	4*x*	15	0.3	0.68	–	18	1.8	–	2	0.133	2	0.133
SAT	4*x*	16	0	0.4	–	14	1.4	–	1	0.063	1	0
SZO	4*x*	15	0.6	0.44	–	21	2.1	–	6	0.4	5	0.81
TAT	4*x*	13	0.7	0.51	–	24	2.4		4	0.308	2	0.731
TOK	4*x*	21	0.3	0.41	–	17	1.7	–	4	0.19	4	0.614
TOT	4*x*	9	0.5	0.26	–	17	1.7	–	2	0.222	2	0.389
VIL	4*x*	7	0.4	0.21	–	14	1.4	–	4	0.571	4	0.714
P. rothiana												
BAG	4*x*	7	0	0.7	–	17	1.7	–	1	0.143	1	0
BOD	4*x*	14	0.5	0.73	–	23	2.3	–	7	0.467	5	0.868
CSA	4*x*	15	0.1	0.8	–	23	2.3	–	4	0.267	1	0.667
HAJ	4*x*	15	0	0.9	–	21	2.1	–	1	0.067	1	0
JUR	4*x*	17	0.6	0.69	–	19	1.9	–	2	0.118	2	0.221
KAD	4*x*	17	0.8	0.73	–	24	2.4	–	5	0.294	3	0.728
LES	4*x*	7	0	0.7	–	17	1.7	–	1	0.143	1	0
PIL	4*x*	7	0.2	0.59	–	16	1.6	–	2	0.286	1	0.571
SUT	4*x*	11	0.1	0.8	–	18	1.8	–	2	0.182	2	0.509
SZR	4*x*	8	1	0.7	–	17	1.7	–	2	0.25	2	0.25
VAR	4*x*	8	0	0.7	–	17	1.7	–	1	0.125	1	0

For population codes see Table 1

PL ploidy level, *N* sample size, *P* percentage of polymorphic loci, H_o observed heterozygosity, H_e expected heterozygosity (in diploids only, calculated according to Nei 1973), ΣA sum of alleles, *A* mean number of alleles per locus, F_{IS} Wright′s fixation index for population over loci (in diploids only), *G* number of multilocus genotypes, *G/N* proportion of distinguishable genotypes, G_{uni} number of unique genotypes, *D* genotype diversity

Table 4 Summary of genetic variation for 10 loci in *Pilosella echioides* (diploid 2*x* and apomictic 4*x*) and *P. rothiana* (apomictic 4*x*)

	PL	*N*	*P* (*a*)	H_o (*a*)	$\sum A$ (*t*)	$\sum A$ (*a*)	*A* (*a*)	*G* (*t*)	*G* (*a*)	*G/N* (*t*)	*G/N* (*a*)	G_{uni} (*t*)	G_{uni} (*a*)	*D* (*a*)
P. echioides 2*x*	2*x*	58	0.350	0.121	18	15.75	1.575	15	5.75	0.259	0.416	15	2.250	0.828
P. echioides 4*x*	4*x*	135	0.318	0.412	31	17.0	1.7	28	3.5	0.207	0.252	27	2.364	0.387
P. rothiana	4*x*	127	0.300	0.731	31	19.27	1.927	23	1.75	0.181	0.213	22	1.818	0.347

PL ploidy level, *N* sample size, *P* percentages of polymorphic loci, H_o observed heterozygosity, ΣA sum of alleles, *A* mean number of alleles per locus, *G* number of multilocus genotypes, *G/N* proportion of distinguishable genotypes, G_{uni} number of unique genotypes, *D* genotype diversity, *t* total, *a* averaged across samples (populations)

of the diversity, whereas intra-population variation is low (mean of 2.64 genotypes in *P. echioides* and 2.55 in *P. rothiana*). Several populations are uniclonal (three in *P. echioides*, four in *P. rothiana*). Only 7.14 % of genotypes of *P. echioides* and 21.74 % of genotypes of *P. rothiana* were detected in more than one population. This distribution of diversity is rather surprising and raises questions related to processes leading to the observed pattern. In *P. echioides*, tetraploid apomictic populations are mostly located in a 50 × 40 km region in northernmost Hungary. Based on the richness of herbarium material and literary data from the study area, the species was once rather common and underwent a decline after World War II. We can imagine a higher frequency of genetically diverse and differentiated diploid populations, and recurrent polytopic origins of autotetraploids. That these tetraploids are apomictic nevertheless remains a weak point of this hypothesis, as a common and recurrent shift from

Table 5 Genome sizes (2*C* values in DNA pg; mean ± SD) of nine diploid *Pilosella* samples from the locality Lesenceistvánd (Hungary, Veszprém county), determined using propidium iodide flow cytometry

Taxon	2*C*
P. leucopsilon	3.84 ± 0.01 ($n = 3$)
P echioides	4.68 ± 0.02 ($n = 2$)
Hybrid (*P. sterrochaetia*)	4.24 ± 0.03 ($n = 4$)
Theoretical intermediate value between parents	4.26

sexuality to apomixis would be necessary. There is an alternative explanation, however: it is possible that numerous tetraploid apomictic clones were widely distributed in the past. These clones might have arisen from independent polyploidization events and accumulated further diversity through residual sexuality, which is common in the genus *Pilosella*. Locally different selection pressures could then have led to geographically structured diversity. However, the most plausible explanation, which is also supported by our data, supposes a monophyletic origin of north-Hungarian apomictic tetraploids—all but two populations (NYE and TOT) form a separated cluster. A shift to apomixis in sexual tetraploids (which could have arisen from diploids via unreduced gametes) should also be considered. One possible scenario might include hybridization between genetically different sexual tetraploids and a shift to apomixis due to lower compatibility of the contributing genomes. Last but not least, it can be hypothesized that apomictic tetraploids are not autopolyploids and that they have been affected by introgression from already apomictic plants. As regards the origin of *P. echioides* polyploids, twelve alleles detected in tetraploids are, interestingly, not present in diploids. All but three (*6Pgdh1-d*, *Shdh-b*, *Dia2-a*) occur at extremely low frequencies, however, the three alleles are also far from being ubiquitous in tetraploids. It is probable that mutations and introgression (from *P. piloselloides* or its relatives) took place. Morphological variation suggests that introgression might have played a role in the populations LEA, SZO, and TAT. We are nevertheless aware that the presence or absence of alleles may be biased by the limited number of populations analysed.

Nearly, the same applies to apomictic *P. rothiana*, which often occurs in habitats more or less influenced or even created by man (road margins, sand pits, disturbed places in grasslands). Such populations are sensitive to succession leading to high-canopy grasslands, and the pattern of distribution is undoubtedly unstable over time. Dispersal by wind is easy because there are no prominent landscape barriers, and at least some of the investigated populations seem to be rather recent. At most localities, *P. rothiana* grows without its putative parental species. A recent in situ origin by hybridization can be excluded or at least unlikely. Under this scenario, one would expect one or a few locally or widely distributed clones. However, the total of 23 clones and the rather high inter-population variation can indicate residual sexual processes or, less likely, a polytopic origin. We found especially high genotype diversity in the populations BOD and KAD. At these two localities, *P. rothiana* grows together with diploid *P. leucopsilon*. Although we collected the plants for the current study with special care to avoid putative hybrids, a certain level of introgression, not apparent in the morphology, cannot be fully excluded. On the other hand, we did not observe any plants that would clearly be intermediate between *P. rothiana* and *P. leucopsilon*.

The allozyme pattern in *P. rothiana* seems to be in some aspects congruent with morphology. Plants with long and dense simple eglandular hairs on involucral bracts and a distinct branching pattern of inflorescences (one flower head consistently overtopped the others in all populations) from the vicinity of Veszprém and the southern part of the Vertés Mts. form a separate group within the *P. rothiana* cluster (populations CSA, HAJ and KAD). These plants correspond to *P. rothiana* subsp. *balatonense* (Borb.) [*H. rothianum* subsp. *balatonense* (Borb.) Zahn], described from this region.

The genetic variation in tetraploid *P. echioides* and *P. rothiana* is comparable to that in other polyploid and apomictic *Pilosella* species or cytotypes. It is not easy to compare it with that of polyploid apomicts from other genera because the mechanisms of apomixis often differ. Such comparisons lie beyond the scope of this paper. In some aspects, similar genetic diversity has been found in *P. floribunda*, a stabilized hybridogeneous apomictic tetraploid from the Czech Republic with one to several different clones in particular mountain ranges (Fehrer et al. 2005). However, the individual mountain ranges in the case of *P. floribunda* have at least recently been isolated from each other. This cannot be said for *P. rothiana* in northern Hungary. The species more probably originated from multiple independent hybridizations between local clones. Genetic variation in polyploid 'basic' *Pilosella* taxa strongly varies among species and partly also among geographic areas. Different species show entirely different patterns. In *P. aurantiaca*, one tetraploid clone is widely distributed throughout Central Europe and has also been introduced to North America; however, high population-level variation has been discovered in tetra- and pentaploid populations in the Eastern Carpathians. Extremely low genetic variation (one tetra- and one pentaploid clone) has been detected in *P. caespitosa* (Fehrer et al. 2005). By contrast, molecular studies of penta- and hexaploid *P. bauhinii* have revealed high genetic variation at both the intra- and inter-population level (Krahulcová et al. 2009). Similar

variation has also been detected in allotetraploid *P. alpicola* s.str. (Šingliarová et al. 2011). Rather high genetic variation has also been reported for Nordic *Pilosella* populations (Tyler 2005). Genetic variation in *Pilosella* species obviously strongly varies among species and cytotype in different geographic regions.

Delimitation of *P. echioides* and *P. rothiana*—congruence between allozyme diversity and morphology

Although most plants or populations of *P. rothiana* are rather easily distinguishable from *P. echioides* using morphological characters (especially the architecture of the inflorescence, indumentum type and number of stem and rosette leaves), problems can arise when plants not clearly belonging to any of these species are encountered. We detected several alleles unique to *P. rothiana* (as opposed to *P. echioides*). Some alleles, however, occur only at (very) low frequencies and are of limited use for reliable delimitation of the two species. The most useful is allele *Aat3-a*, detected consistently in *P. rothiana* plants and also consistently present (in homozygous state) in *P. officinarum* (Chrtek et al., unpubl.), which clearly confirms the hybrid origin of *P. rothiana*. Both species also distinctly differ in their nuclear DNA content (Suda et al. 2007).

Origin of species intermediate between *P. echioides* and P. *officinarum*/leucopsilon

While the origin of some intermediate *Pilosella* species in certain groups has been explained satisfactorily (e.g., Krahulec et al. 2004, 2008), the origin of *P. echioides*–*P. leucopsilon*/*officinarum* intermediates remains a puzzle. In this study, we show that diploid *P. echioides* and *P. leucopsilon* can hybridize in nature. Hybrid plants are diploid, and their reproductive potential (male and female fertility) needs to be examined further. It is much more difficult to trace the evolutionary pathways leading to types morphologically closer to *P. echioides*, i.e., *P. rothiana* and *P. erythrodonta*. Firstly, it is notoriously hard to assign a plant to a hybrid combination based on morphology alone. Zahn (1921–1923, 1922–1930) suggested, as a distinguishing feature, the colour of the tip of outer ligules (red in *P. erythrodontia*, yellow in *P. rothiana*), but this does not seem to be a reliable character. We did not observe red-tipped outer ligules in any of the plants encountered during the course of this study. Not even careful inspection of herbarium material (especially in BP) has helped us solve this problem. Secondly, there are multiple ways of attaining tetraploid plants (named here as *P. rothiana*). Several possible combinations of reduced and unreduced gametes of *P. echioides* on one side and *P. officinarum* or *P. leucopsilon* on the other exist. The participation of both reduced and unreduced gametes in *Pilosella* crosses has been confirmed repeatedly (Fehrer et al. 2007; Krahulcová et al. 2009). Crossing experiments might cast more light on this topic. We can only speculate that the tetraploid (not diploid) cytotype of *P. echioides* participated in the origin of *P. rothiana*. At least preliminarily, we assign all our *P. echioides* > *P. leucopsilon*/*officinarum* plants to *P. rothiana* (*P. echioides* > *P. officinarum*).

A hybrid of the same parental combination as our diploid hybrid (*P. echioides* < *P. leucopsilon*) has been given the name *P. sterrochaetia*. The species was described based on a single herbarium specimen (preserved in W) from the collection of Rochel but without indication of who collected it and where (Nägeli and Peter 1885). It has been supposed that the plant originated from Banat ('Banat?' in Zahn 1921–1923; 1922–1930). Zahn (1921–1923; 1922–1930) also reported its very rare occurrence in Lower Austria (Niederösterreich), the southeastern shore of the Black Sea (historical Lazistan) and the Caucasus Mts. The species had not been recently reported from the Hungarian territory (Zahn 1921–1923; Zahn 1922–1930; Soó 1970). Our find is thus the first for the country. However, some plants assigned in the Hungarian Flora (Soó 1970) to the morphologically very similar *P. bifurca* (*P. echioides* ≤ *P. officinarum*) might belong to *P. sterrochaetia* (proper identification is difficult in herbarium specimens). Chromosome counting might be a helpful tool for deciding between the two putative parent species (*P. leucopsilon* vs. *P. officinarum*). Mráz et al. (2008) reported *Pilosella officinarum* from northeastern Hungary and southern Slovakia to be predominantly pentaploid, and its hybrids with *P. echioides* could hardly be diploid. Diploid plants of *P. echioides* < *P. leucopsilon*/*officinarum* can thus be assigned to the hybrid combination *P. echioides* (2*x*) × *P. leucopsilon* (2*x*). Another possible pathway to tetraploids is the participation of the apomictic tetraploid *P. echioides* in crosses with *P. leucopsilon*. These crosses would most probably produce triploid or pentaploid hybrid plants (in this case, the same ploidy could result from certain *P. echioides* × *P. officinarum* crosses). In any case, recent diploid *Pilosella* hybrids are very rare. Although some of these hybrids have been proven to be fertile, their rarity in nature probably strongly limits their evolutionary significance (Krahulec et al. 2004).

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