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Biosystematic study of the diploid-polyploid *Pilosella alpicola* group with variation in breeding system: Patterns and processes

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Abstract Members of the *Pilosella alpicola* species group (Asteraceae) are distributed throughout the alpine region of the European mountains (Alps, Carpathians, Balkan mountains). Like other *Pilosella* species groups (*Hieracium* subg. *Pilosella*), the taxonomy and species' relationships are poorly understood mostly due to widespread facultative apomixis, frequent hybridization and polyploidization—the most important phenomena substantially involved in the evolutionary history of the genus. We assessed morphology, ploidy level, variation in breeding system and molecular variation within the *P. alpicola* group to provide a new taxonomic concept and to clarify evolutionary relationships among species and origin of polyploids. Multivariate morphometric analyses (UPGMA, CDA, PCA) applied on 324 plants originated from 21 populations revealed existence of four well-separated clusters corresponding to four allopatric taxa: *P. alpicola* s.str. (Alps), *P. rhodopea* (Balkan Peninsula, Southern Carpathians), *P. serbica* (Serbia and Montenegro) and *P. ullepitschii* (Carpathians). In total, four ploidy levels (2*x*, 3*x*, 4*x*, 5*x*) were detected among 557 plants analysed from 19 populations by classical chromosome counting and flow cytometric analysis. The tetra- and pentaploids of *P. alpicola* s.str. have an allopatric distribution (Wallis Alps vs. Dolomites, respectively). Four ploidy levels with complex cytogeographic pattern and high frequency of mixed ploidy populations (75%) indicating a primary contact zone were recorded in *P. rhodopea*. *Pilosella ullepitschii* and *P. serbica* are exclusively diploid and are both sexually reproducing. In spite of a clear morphological separation, a molecular analysis (ITS and cpDNA sequences) suggests close relationships and rather recent origin of all studied taxa, except *P. alpicola* s.str. The latter taxon is an agamospermic allopolyploid that likely originated polytopically from a hybridization between *P. rhodopea* from the Balkans and *P. glacialis* from the Alps. In contrast to *P. alpicola* s.str., our data strongly support an autopolyploid origin of *P. rhodopea* polyploids which reproduced strictly sexually. *Pilosella petraea,* sometimes treated as a member of the *P. alpicola* group, differs from the remaining taxa by its conspicuous morphology, ecology and ITS polymorphism and should be removed from the group. Range shifts and extinctions were likely involved in shaping the evolutionary and modern distributional pattern of the group. Our combined methodological approach enabled us to propose a new taxonomic circumscription for the *P. alpicola* group and revealed auto- and allopolyploidization events.

Keywords allopatric speciation; allopolyploidy; apomixis; autopolyploidy; biogeography; flow cytometric seed screening analysis; *Hieracium*; ITS; multivariate morphometrics

Supplementary Material Tables S1 and S2, and Figures S1 and S2 are available in the free Electronic Supplement to the online version of this article

INTRODUCTION

The genus *Pilosella*, often treated as a subgenus of *Hieracium* L., belongs to the taxonomically most intricate vascular genera of the Northern Hemisphere. Hybridization, polyploidization and versatile breeding systems have all been considered key evolutionary mechanisms in the genus (e.g., Krahulcová & al., 2000; Fehrer & al., 2007b). These phenomena have largely contributed to the taxonomic complexity and thus have so far obscured species relationships in *Pilosella*. Thus, detailed biosystematics studies are needed to improve taxonomic understanding of the genus. We performed a detailed study of the *Pilosella alpicola* group using various methodological approaches with the aim to solve the taxonomic problems and to clarify the role of polyploidization and hybridization in the evolution of this group.

The reticulate pattern of morphological variation in the genus *Pilosella* has resulted in several, in some cases contradictory, taxonomic concepts (Nägeli & Peter, 1885; Zahn, 1922–1930; Sell & West, 1976; Tyler, 2001; Schuhwerk, 2002; Krahulec & Krahulcová, 2006; Bräutigam & Greuter, 2007). The most common species concept is based on the recognition of so-called "basic" and "intermediate" species (Nägeli & Peter, 1885). Species are characterized as basic (*species principales*) if they have unique phenotypic traits and intermediate (*species intermediae*) if they have a combination of characters from two or more basic species. This classification system has resulted in the identification of ca. 160 species, and many infraspecific taxa within both basic and intermediate species (Zahn, 1922–1930). Large ploidy level variation is characteristic for the genus; the ploidy ranges from diploid (2*x*) to octoploid (8*x*), with basic chromosome number *x* = 9

(Schuhwerk, 1996). In general, basic species are either diploid or polyploid, while intermediate species are usually polyploid (Schuhwerk, 1996); however, many taxa exhibit infraspecific cytotype variation. The asexual formation of the seeds was recorded in polyploids as early as 1907 (Rosenberg, 1907) and was later characterized as aposporous apomixis of the *Hieracium* type with autonomous endosperm formation (Pogan & Wcisło, 1995). Diploids are strictly self-incompatible, however an induced autogamy was occasionally recorded in interspecific crosses (Krahulcová & al., 1999). Polyploids are usually (facultatively) apomictic, but even they (4*x*, 6*x*) often reproduce sexually. However, both breeding systems may operate even within one inflorescence (capitulum). The genus is notorious for widespread interspecific and interploidal hybridization (Krahulcová & al., 2000; Fehrer & al., 2007b).

Morphologically, the *Pilosella alpicola* group (= *P.* sect. *Alpicolinae* (Nägeli & Peter) Szeląg) is characterized by a dwarf habit, the absence of above-ground stolons, and small numbers of inflorescences of relatively large size, which are covered by long and dense indumentum. Ecologically, the members of the group are confined to the primarily alpine and subalpine meadows and screes on acid bedrock and occupy very fragmented ranges in the Pyrenees, Alps, Carpathians and Balkan mountains (Nägeli & Peter, 1885; Zahn, 1922–1930; Bräutigam, 1992). Two different species concepts have been applied for this group. The broader one comprises two species—a Pyrenean *P. breviscapa* DC. (Soják) (= *P. candollei* Monn., nom. illeg.) and *P. alpicola* F.W. Schultz & Sch. Bip. (Zahn, 1922–1930; Sell & West, 1976). Within the latter species, Zahn (1922–1930) distinguished further six subspecies (*Hieracium alpicola* subsp. *alpicola* from the Alps; subsp. *ullepitschii* (Błocki) Zahn and subsp. *furcotae* Degen & Zahn from the Carpathians; subsp. *rhodopeum* (Griseb.) Zahn, subsp. *micromegas* (Fr.) Nägeli & Peter and subsp. *glandulifolium* Nägeli & Peter from the Balkans) and some other taxa at lower ranks. In spite of the existence of several taxa described from different parts of the range, many national floras followed this very broad species concept usually referring to only one taxon—*Pilosella* (*Hieracium*) *alpicola* (Jordanov, 1960; Hess & al., 1972; Zángheri, 1976; Pignatti, 1982; Pawłowski, 1988; Buttler, 1991; Gottschlich & Pujatti, 2002; Szeląg, 2002; Aeschimann & al., 2004). Recently, Szeląg (2008) proposed a narrow species concept and included six taxa at species rank in *P.* sect. *Alpicolinae*: *P. breviscapa*, *P. petraea* F.W. Schultz & Sch. Bip. (= *Hieracium heuffelii* Janka), *P. alpicola* F.W. Schultz & Sch. Bip., *P. rhodopea* (Griseb.) Szeląg, *P. serbica* (F.W. Schultz & Sch. Bip.) Szeląg and *P. ullepitschii* (Błocki) Szeląg. Phylogenetic analysis of the genus *Pilosella* based on ITS sequences showed that at least *P. ullepitschii* (as *P. alpicola* subsp. *ullepitschii*) and *P. breviscapa* are not closely related (Fehrer & al., 2007a). Moreover, our preliminary observations suggested that *P. petraea* does not belong to the *P. alpicola* group. Under these aspects, Zahn's and Szeląg's concepts, based exclusively on morphological observations, deserve a critical revision.

With the exception of the Carpathian populations (Šingliarová & Mráz, 2009), very little is known about karyological and reproductive variation of the *P. alpicola* group. This information, however, is fundamental for the interpretation of morphological and genetic variation, especially in polyploid apomictic complexes. A recent detailed study revealed that 15 Carpathian populations belonging to *P. ullepitschii*, *P. rhodopea* and *P. petraea* were uniformly diploid ($2n = 2x = 18$) (Murín & al., 1999; Šingliarová & Mráz, 2009; Mráz & Šingliarová, 2009). Three cytotypes have been reported so far from only three localities from the remaining part of the range (Alps, Balkans). The tetraploid cytotype ($2n = 4x = 36$) of *Hieracium alpicola* was recorded in the Swiss Alps (Favarger, 1959), the diploids and triploids under the name *H.* resp. *P. alpicola* ($2n = 2x = 18$ and $2n = 3x = 27$) were found in the Pirin Mts (Bulgaria) (Vladimirov & Szeląg, 2001; Krahulcová & al., 2009b) and finally a diploid chromosome number ($2n = 18$) was published for *P. serbica* (Szeląg & al., 2007) from its type locality in Serbia. Concerning the breeding system, sexuality was experimentally ascertained for diploid *P. ullepitschii* (Šingliarová & Mráz, 2009).

The main aim of this study is a taxonomic revision of the *Pilosella alpicola* group. We carried out a large-scale ploidy level survey, analysis of breeding system using flow cytometry seed screening (FCSS), and multivariate morphometric and molecular analyses. More specifically, we aim to answer the following questions: (1) How variable are morphology, ploidy level and breeding system within the *Pilosella alpicola* group and how can we explain this variation? (2) What are the phylogenetic relationships within the *Pilosella alpicola* group? (3) How did the polyploid cytotypes originate?

■ MATERIALS AND METHODS

Sampling. — Plants were collected randomly from their natural habitats in 2005–2009 throughout their distributional range and transplanted into an experimental garden at the Institute of Botany in Bratislava. In addition, we also used herbarium specimens, deposited in the herbarium of the Institute of Botany in Bratislava (SAV) for further morphometric study. The list of localities is given in Table 1. The populations of *P. breviscapa* were not included in our study, as this taxon is phylogenetically not related to *P. alpicola* s.l. (Fehrer & al., 2007a).

Chromosome number and ploidy level determination. — Chromosome counts were performed on somatic mitosis in root-tip meristems of selected cultivated plants using colchicine as a pre-treatment medium (for further details see Mráz & al., 2008). Selected permanent slides were deposited at the Institute of Botany in Bratislava.

Flow cytometry, a rapid and reliable method for accurate estimation of DNA-ploidy level based on differential intensity of fluorescence of cell nuclei in fluid stream (Doležel, 1991), was used to detect ploidy level for most of the plants. Ploidy level was assessed in 370 cultivated plants originating from 19 localities (Table 1). For eleven plants both the exact chromosome number and the ploidy level estimation were determined. Because the analyses revealed a high frequency of populations with mixed cytotypes in the Balkans, we performed additional flow cytometric measurements of 108 herbarized plants (air-dried, 1–2 years old) that originated from mixed-ploidy

Table 1. List of analyzed populations of the *Pilosella alpicola* group.

Code	Taxon / Locality	MF[a]	PL[b] 2*x*	3*x*	4*x*	5*x*	Total/new
	***P. alpicola* s.str.**						
MOR	CH, Wallis Alps, Mt. Monte Moro, N 46°00′57″, E 07°57′58″, 2306 m	15	–	–	28	–	28
SIM1	CH, Wallis Alps, Simplon Pass, N 46°15′00″, E 08°00′50″, 2000–2400 m	9	–	–	51	–	51
SIM2	CH, Wallis Alps, Simplon Pass, Mt. Hübschhorn, 2100–2400 m	–	–	–	27	–	27
PUF	IT, Dolomites, Mt. Puflatsch, N 46°33′07″, E 11°36′47″, 2160 m	10	–	–	–	34	34
SCH	IT, Dolomites, Mt. Schlern, N 46°29′56″, E 11°34′54″, 2019 m	–	–	–	–	16	16
DUR	IT, Dolomites, Val Duron valley, N 46°29′52″, E 11°39′35″, 2236 m	–	–	–	–	17	17
Total		**34**					**173/173**
	P. ullepitschii						
BAR*	SK, Západné Tatry Mts, Trnovecká valley, N 49°09′46.5″, E 19°44′04″, 1885 m	–	21	–	–	–	21
LAL*	SK / PL, Západné Tatry Mts, Ľaliové saddle, N 49°13′35″, E 19°59′30″, 1952 m	4	22	–	–	–	22
KR2*	SK, Vysoké Tatry Mts, Mt. Kriváň, SW, N 49°09′27″, E 19°59′25″, 1900 m	12	20	–	–	–	20
KR5*	SK, Vysoké Tatry Mts, Mt. Kriváň, SE, N 49°09′02″, E 19°59′55″, 1890–1900 m	10	22	–	–	–	22
FUR *	SK, Vysoké Tatry Mts, Furkotská valley, N 49°09′12″, E 20°01′43″, 1910 m	19	37	–	–	–	37
MLY*	SK, Vysoké Tatry Mts, Mlynická valley, N 49°09′30″, E 20°02′30″, 1675–2017 m	8	27	–	–	–	27
MEN*	SK, Vysoké Tatry Mts, Mengusovská valley, N 49°09′57″, E 20°03′40″, 1800–1875 m	19	21	–	–	–	21
OST*	SK, Vysoké Tatry Mts, Mt. Ostrva, N 49°08′58″, E 20°05′22″, 1959 m	4	20	–	–	–	20
BUC*	RO, Bucegi Mts, Cabana Babele, N 45°24′24″, E 25°28′30″, 2160–2204 m	19	41	–	–	–	41
NMA*	RO, Nemira Mts, Mt. Nemira Mare, N 46°15′21.5″, E 26°19′25.5″, 1641 m	–	18	–	–	–	18
NMI*	RO, Nemira Mts, Mt. Nemira Mica, N 46°13′59″, E 26°19′55″, 1619 m	–	6	–	–	–	6
SMA*	RO, Nemira Mts, Mt. Sandru Mare, N 46°11′57″, E 26°20′21″, 1590–1640 m	–	48	–	–	–	50
Total		**95**					**305/0**
	P. rhodopea						
KOR	AL, Korab Mts, Mt. Maja e Korabit, N 41°48′00″, E 20°31′38″, 1920 m	20	13	11	11	–	35
MUS	BG, Rila Mts, Mt. Jastrebets, N 42°13′29″, E 23°34′46″, 2359 m	19	–	6	23	7	36
DOD	BG, Rila Mts, Mt. Dodov vrah, N 42°09′59″, E 23°20′23″, 2540 m	20	25	2	–	–	27
MAL	BG, Rila Mts, Maljovitsa hut, N 42°11′22″, E 23°22′28″, 1985 m	–	–	4	–	–	4
GRA	BG, Rila Mts, Granchar valley, N 42°07′16″, E 23°35′29″, 2200 m	20	–	25	4	–	29
GRA-W	BG, Rila Mts, Granchar valley, Dzhanka ridge, N 42°07′16″, E 23°35′29″, 2342 m	–	8	31	15	–	54
VEZ	BG, Stara planina Mts, Mt. Vezhen, N 42°45′35″, E 24°23′45″, 2130 m	18	15	–	–	–	15
BOT	BG, Stara planina Mts, Mt. Botev, N 42°42′54″, E 24°55′01″, 2352 m	20	7	16	16	1	40
TAZ	BG, Stara planina Mts, above the Taza chalet, N 42°41′30″, E 24°40′00″, 2050 m	–	3	5	4	–	12
VICH	BG, Pirin Mts, Mt. Vichren, N 41°45′39″, E 23°24′27″, 2331 m	20	–	31	3	–	34
BEZ	BG, Pirin Mts, Mt. Bezbog, N 41°43′35″, E 23°30′57″, 2414 m	20	–	34	1	–	35
COZ*	RO, Cozia Mts, Mt. Cozia, N 45°19′04″, E 24°20′17″, 1592 m	20	14	–	–	–	14
Total		**177**					**335/321**
	P. serbica						
KOP	SR, Kopaonik Mts, Mt. Suvo Rudishte, N 43°16′28″, E 20°48′55″, 1917 m	**18**	**63**	**–**	**–**	**–**	**63/63**
Total		**324**					**876/557**

Abbreviations for countries: AL, Albania; BG, Bulgaria; CH, Switzerland; IT, Italy; PL, Poland; RO, Romania; SK, Slovakia; SR, Serbia.
Symbol "*" denotes the populations for which the DNA-ploidy level and/or chromosome numbers were published by Šingliarová & Mráz (2009).
[a]MF, number of plants used for morphometric study.
[b]PL, number of plants studied karyologically and/or measured for the DNA-ploidy level (total/new).

populations. Ploidy level estimations failed in ca 20% of cases most likely due to the disintegration of plant tissues during drying process and subsequent storing of herbarium vouchers. In addition, we estimated the ploidy level for 46 herbarized plants not used in the morphometric analyses. Ploidy level was also estimated for 33 mother plants and 181 seeds in order to detect the mode of reproduction using flow cytometric seed screen analysis (FCSS, see below). In total, we detected the ploidy level for 557 plants and 181 seeds.

Samples from living plants and herbarium vouchers were prepared using a two-step procedure (Otto, 1990; Doležel & Göhde, 1995). For details on sample preparations see Mráz & al. (2008). The clones of previously cytologically analysed diploid plants of *Pilosella lactucella* (Wallr.) P.D. Sell & C. West ($2n = 2x = 18$) were used as an internal reference standard for ploidy level estimations of living individuals, while the DNA-ploidy levels of herbarium specimens were assessed using *Bellis perennis* L. (2C = 3.38 pg, Schönswetter & al., 2007b). Flow cytometric analyses of cultivated plants were performed with a FACSCalibur instrument (Becton Dickinson) equipped with an argon-ion laser exciting at 488 nm at the Institute of Biology and Ecology, P.J. Šafárik University in Košice. Propidium iodide (PI) was used as a stain. The ploidy level of herbarium specimens was detected by a Partec Cyflow instrument equipped with a HBO lamp at the Institute of Botany, Slovak Academy of Sciences, Bratislava with 4,6-diamino-2-phenylindole (DAPI) as a stain. Histograms were accumulated at flow rate of about 20–50 particles per second for a total count of 3000–5000 nuclei. Only measurements with coefficients of variation up to 5% for fresh material (PI staining) or 10% for herbarium vouchers (DAPI staining) were used for analysis.

Breeding system detection. — The breeding system in Lactuceae genera with co-occurring sexual and asexual reproduction can be easily inferred from a combination of isolation, castration and open pollination experiment (Richards, 1997). However, the number of plants of the alpine *P. alpicola* group flowering at lowland experimental conditions was very low. Therefore, we performed flow cytometry seed screening analysis (FCSS, cf. Matzk & al., 2000) for study of reproductive pathways in *P. alpicola* s.str., *P. rhodopea* and *P. serbica*. This method is based on the comparison of the ploidy of embryo and corresponding endosperm within one seed. In the case of sexually formed seed (gamete fusion), the ratio between relative fluorescence of endosperm and embryo peaks is expected to vary around 1.5 because of a diploid embryo and a triploid endosperm (depending on the ploidies of ovule and pollen involved), while in the case of autonomous apomixis (e.g., Koltunow, 1993; Noyes, 2007) in the genus *Pilosella* this ratio is exactly 2.0. Moreover, the FCSS method allows the identification of the ploidy of gametes that participated on embryo formation when the ploidy level of the maternal (seed) plant is known. FCSS analysis was applied on individual seeds to accurately discriminate both embryo and endosperm peaks. Ripe achenes were collected from the plants from following natural populations: GRA-W, KOP, MOR, PUF and SIM1 (see Table 1). The ploidy of the mother plant was estimated from the dried-up part of involucral bracts and/or stems. The seed samples and the samples from the maternal plant were prepared in a two-step procedure (see above). Each seed was measured separately and twice—without standard and after addition of standard. We used the leaves of diploid *P. lactucella* as a reference standard. Relative fluorescence intensity of DAPI stained nuclei (1500–5000 particles) was measured by Partec Cyflow instrument equipped with a HBO lamp.

Morphometric analysis. — Multivariate morphometrics (Sneath & Sokal, 1973) has been successfully used in several taxonomically difficult genera with widespread apomictic reproduction (e.g., *Crataegus*: Smith & Phipps, 1988; *Alchemilla*: Sepp & Paal, 2000; *Ranunculus*: Hörandl & al., 2009; *Sorbus*: Lepší & al., 2009), including the closely related genus *Hieracium* (Chrtek & al., 2007; Tyler, 2006). Surprisingly, as far as we know this approach has never been applied in the genus *Pilosella*, except still unpublished work of Urfus (Urfus, 2006).

For the purpose of morphometric comparison we measured selected morphological characters on 324 herbarized plants from 21 populations (Table 1). A population sample usually included 10–20 flowering individuals. In some cases the sample size, however, was lower than ten plants per population because of a small number of flowering plants. Samples from known type localities of traditionally recognized taxa were also included in our study. Morphological characters measured or scored are those used for identifying taxa of the *P. alpicola* group (Zahn, 1922–1930; Szeląg, 2008) and some new potentially useful were added. Forty-three characters (26 quantitative, 12 semiquantitative, one binary, and four ratios derived (Table 2; Fig. 1; Fig. S1) were measured or scored on herbarized plant material. Ligules and involucral bracts were attached to paper by adhesive tape immediately in the field, then scanned and measured by CARNOY v.2.0 (Schols & al., 2002).

Five datasets were used in the analyses:

1. Twenty-one population samples of the *P. alpicola* group from the whole area characterized by the mean values of all 43 characters as OTUs (operational taxonomic units) (matrix A).
2. A complete, pooled dataset including all 43 characters and 324 individual plants from 21 populations covering the whole area as OTUs (matrix B).
3. A dataset consisting of 139 plants from the Balkans and Cozia Mts with detected ploidy level (61, 2*x*; 54, 3*x*; 22, 4*x*; 2, 5*x*) as OTUs and 42 characters (excluding LibDS—invariable across the group) (matrix C).
4. A dataset consisting of 34 plants from the Alps as OTUs and all 43 characters (matrix D).
5. A data matrix including all 324 plants from the whole area as OTUs and 26 quantitative and 4 ratio characters only (matrix E).

Pearson (parametric) and Spearman (non-parametric) correlation coefficients were computed for all data matrices to study the relationships between particular variables. As no character pair showed strong correlation potentially distorting further computations, all 43 (matrices A, B, D), 42 (matrix C) and 30 (matrix E) characters were used in further analyses. We performed both hierarchical and non-hierarchical multivariate evaluation of the morphological data in the following steps:

Table 2. Morphologic characters used for morphometric analyses of the *Pilosella alpicola* group.

Characters[a]
LNo – number of rosette leaves
LoL – length of leaves from outer part of rosette (hereafter outer leaves) (cm)[b]
LoW – maximum width of outer leaves (cm)[b]
LiL – length of leaves from inner part of rosette (hereafter inner leaves) (cm)[b]
LiW – maximum width of inner leaves (cm)[b]
LoDT – density of simple eglandular trichomes on adaxial side of outer leaves[c]
LiDT – density of simple eglandular trichomes on adaxial side of inner leaves[c]
LoLT – length of simple eglandular trichomes on adaxial side of outer leaves (mm)[d]
LiLT – length of simple eglandular trichomes on adaxial side of inner leaves (mm)[d]
LoDS – density of stellate trichomes on adaxial side of outer leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LiDS – density of stellate trichomes on adaxial side of inner leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LobDS – density of stellate trichomes on abaxial side of outer leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LibDS – density of stellate trichomes on abaxial side of inner leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LbDG – density of glandular trichomes on abaxial side of leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LmDG – glandular trichomes at the margins of leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LSNo – number of cauline leaves
LSL1 – length of the lowest stem leaf (cm)
LSL2 – length of the second lowest stem leaf (cm)
SL – stem length (from basis to terminal capitulum) (cm)
SLL – stem length up to the lowest stem leaf (cm)
SDT – density of simple eglandular trichomes on peduncle (0 – a few, 1 – numerous, 2 – dense)[e]
STL – length of simple eglandular trichomes on peduncle (mm)
STC – colour of simple eglandular trichomes on peduncle (0 – dark up to the middle, 1 – dark more than to the middle)[f]
SDS – density of stellate trichomes on peduncle (0 – a few, 1 – sparse, 2 – dense)[e]
SDG – density of glandular trichomes on peduncle (0 – a few, 1 – sparse, 2 – dense)[e]
CNo – number of well-developed capitula (capitula with flowers)
CaNo – number of undeveloped (aborted) capitula
AL – length of acladium of terminal capitulum (cm)
INo – number of involucral bracts of terminal capitulum
IL – length of involucral bracts of terminal capitulum (mm)[g]
IW – width of involucral bracts of terminal capitulum (mm)[g]
ITL – length of simple eglandular trichomes on involucral bracts (mm)[g]
ITC – colour of simple eglandular trichomes on involucral bracts (0 – predominantly pale, in some cases with dark basis, 1 – dark approximately to the middle, 2 – predominantly dark, eventually with pale apex)[f]
IC – colour of involucral bracts (0 – silver grey, 1 – grey, 2 – grey-black)
IDT – density of simple eglandular trichomes on involucrum (0 – a few, 1 – sparse, 2 – dense)
FNo – number of ligules in terminal capitulum
FL – length of ligule in terminal capitulum (mm)[g]
FW – width of ligule in terminal capitulum (mm)[g]
StL – length of styles in terminal capitulum (mm)[g]
LoL/LoW
LiL/LiW
FW/FL
StyL/FL

[a] See also Fig. 1 and Fig. S1.
[b] Mean values of the two largest leaves.
[c] Mean number of eglandular trichomes on the two largest leaves, counted on 0.25 cm² in the central part using paper template.
[d] Mean value of three randomly selected trichomes from one leaf.
[e] Estimated, see Fig. S1.
[f] pale – white, ivory, yellowish, silver grey; dark – smoky grey, grey-black, dark brown-grey
[g] Mean values from the three measurements per individual.

1. **Cluster analyses** (Everitt, 1986) with different algorithms, UPGMA (unweighed pair-group method using arithmetic averages), complete linkage cluster analysis and Ward's method of clustering (minimization of the increase of error sum of squares), were performed on **matrix A** to get first insight into the phenetic relationships among all studied populations of the *P. alpicola* group. The characters in the primary matrix were standardized by zero mean and unit standard deviation and the Euclidean coefficient was used to compute the secondary distance matrix.

2. Differentiation suggested by cluster analyses (four entities, see Results) was tested by canonical **(CDA1)** and nonparametric classificatory discriminate **(NCD)** analyses (Klecka, 1980; Krzanowski, 1990) based on the **matrix B.** To reveal correlation of characters with the canonical axes, total canonical structure was computed. The NCD was used to assess the percentage of plants correctly assigned to the predetermined groups.

3. **PCA** based on **matrix C** was used to infer potential morphological differentiation among different cytotypes of plants originated from the Balkans and Cozia Mts in Romania.

4. Morphological consistency of two cytotypes of *P. alpicola* s.str. from two disjunct ranges (tetraploids from Walliser Alps and pentaploids from Dolomites) was tested by principal coordinate analysis **PCoA** using Gower's coefficient for mixed data (Podani, 2001) because the number of OTUs exceeds the number of characters, based on **matrix D.**

5. Because Zahn (1922–1930) and Szeląg (2008) used mostly semi-quantitative or qualitative characters to distinguish relevant taxa from the *Pilosella alpicola* group, we ran **CDA2** on **matrix E** to assess a taxonomic significance of quantitative characters for distinguishing morphological entities revealed by cluster analyses and CDA1.

6. Descriptive data analysis was used to obtain basic statistics of quantitative characters and ratio (mean, percentile 10% and 90%, and standard deviation) for each taxon revealed. For semi-quantitative and binary characters the frequencies of particular states are presented.

All analyses were carried out using SAS v.9.2; only cluster analyses and PCoA were computed in SYN-TAX 2000 (Podani, 2001).

DNA extraction and sequencing. — We selected the internal transcribed spacer (ITS) of ribosomal nuclear DNA to test monophyly and relationships within the *Pilosella alpicola* group as ITS sequences were more informative in the genus *Pilosella* than plastid *matK* and *trnL-trnF* introns (Fehrer & al., 2007a). Moreover, ITS is a suitable marker to unravel

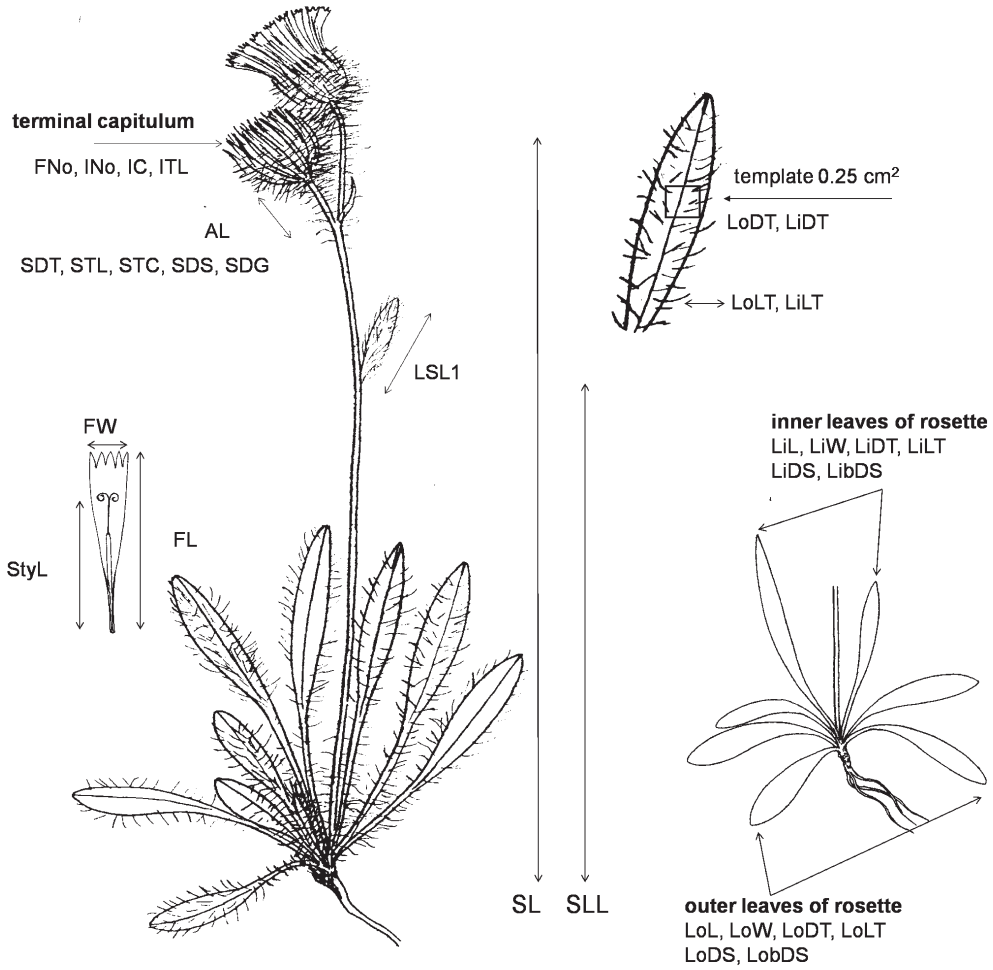

Fig. 1. Illustrations of selected morphological characters measured in the *Pilosella alpicola* group.

hybridization and allopolyploidization events, if the process of homogenisation is very slow or completely absent (e.g., Fehrer & al., 2009). In addition, we tested the plastid *rps16-trnK* spacer for polymorphism. Timme & al. (2007) recently reported that this locus belongs to the most divergent in Asteraceae and that it might be potentially suitable for species-level phylogeny. Moreover, according to M. Ronikier (pers. comm.) this marker showed intraspecific polymorphism in the closely related *Hypochaeris uniflora*.

In total, we sequenced 21 plants of *P. alpicola* s.l. from 15 populations (see Tables 1 and 6; Table S1) for ITS1–2 (GenBank accession numbers HM627292–627314, except of HM627297 and HM627306). We included also one plant of *P. petraea* from its type locality (accession number HM627306, Ciclova Montană in Banat Mts, Romania), because this taxon was considered a member of *Pilosella* sect. *Alpicolinae* by Szeląg (2008) (see Introduction), and one diploid accession of *P. cymosa* (accession number HM627297, Primovce, Slovakia, cf. Mráz & Šingliarová, 2009), as this species was considered by Zahn (1922–1930) as one of the putative parental taxa of *P. petraea*. The leaves were sampled from cultivated plants with known ploidy level, dried in silica gel and stored at room temperature. Total DNA was extracted from 10–15 mg of silica-dried leaf tissue with the DNeasy 96 Plant Kit (Qiagen), following the manufacturers protocol. The ITS1 and ITS2 spacers were amplified using the primers ITSA (GGAAGGAGAAGTCGTAACAAGG) and ITSB (CTTTTCCTCCGCTTATTGATATG) (Blattner, 1999) in a 25 µl volume containing 1 µl of genomic DNA (10–35 ng), 2.5 µM buffer (10× Buffer II, Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 0.2 µM of each primer, 0.25 µM BSA and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycle profile included the initial denaturation at 95°C/10 min followed by 28 cycles of 95°C/30 s, 94°C/30 s, 52°C/30 s, 72°C/1 min, and ended with 72°C/10 min and 4°C thereafter. PCR products were purified using QIAquick Gel Extraction Kit. Sequencing was performed in both directions using BigDye Terminator v.3.1 (Applied Biosystems). In total, three plants from geographically distant localities (SIM1, DOD, KOP; see Table 1), were tested for polymorphism in the *rps16* gene and the *rps16-trnK* intergenic spacer. We used the same PCR conditions and specific primers as given in Timme & al. (2007). The samples were run on an ABI PRISM 3100 Genetic Analyser. Sequences were assembled and edited using Seqscape v.2.5.0 (Applied Biosystems).

Heterozygous sites (intra-individual polymorphic nucleotide site) in ITS1 and ITS2 were scored on both forward and reverse strains according to Aguilar & Feliner (2003), with exception of "at least 25% of strength of the weakest signal criteria". In some cases we accepted a lower threshold in one of two strands. In order to compare our sequenced material with other taxa from *Pilosella*, we conducted an alignment with MEGA v.4 (Tamura & al., 2007) adding 16 *Pilosella* species with already known ITS sequences (GenBank accessions AJ633390, AY879161, AJ633406, AJ633504, AJ633389, AJ633396, AJ633397, AJ633398, AJ633402, AJ633403, AJ633399, AJ633405, AJ633400, AJ633393, AJ633394, all from Fehrer & al., 2007a, and AY879158 from Schuhwerk, unpub.).

■ RESULTS

Ploidy level variation and cytotype pattern. — Four ploidy levels, varying from diploid to pentaploid, were found in the *Pilosella alpicola* group (Table 1; Figs. 2 and 3).

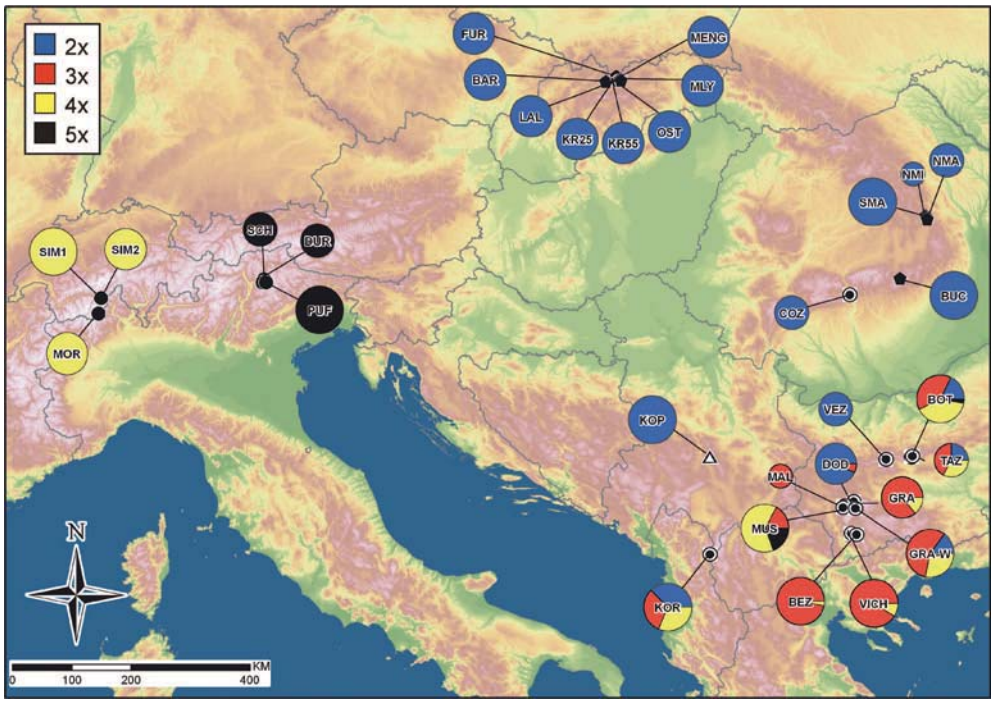

Fig. 2. Cytogeographic pattern in the *Pilosella alpicola* group. *Pilosella alpicola* s.str. (black circles), *P. ullepitchii* (black pentagons, data based on the results published by Šingliarová & Mráz 2009), *P. rhodopea* (double circles, DNA-ploidy level of COZ population published by Šingliarová & Mráz 2009), *P. serbica* (open triangle). Population codes are given in Table 1. Circle size represents four classes of sample size: 1–9, 10–19, 20–30 and more than 30 individuals analysed per population.

The nominate taxon *P. alpicola* s.str. included allopatrically distributed tetra- (4*x*) and pentaploid populations (5*x*). While solely tetraploid cytotypes occured in the Swiss Alps (106 plants analysed from three populations—SIM1, SIM2 and MOR), the pentaploids represented the only cytotype detected in the Italian Alps (67 plants from SCH, PUF and DUR). In total, four ploidy levels (2*x*, 3*x*, 4*x*, 5*x*) were detected in *P. rhodopea*. From 335 plants originating from 12 populations (including the published data on 14 plants from the Cozia population, Šingliarová & Mráz, 2009), 85 were diploid (25.4%), 165 were triploid (49.3%), 77 were tetraploid (23%) and 8 were pentaploid (2.4%). Coexistence of two or more cytotypes was found in 9 out of 12 analysed populations. In one population (BOT) we found all four ploidies. The diploids of *P. rhodopea* were more frequent (52.2%) in the northern part of the species range (Cozia Mts, Stara planina Mts), but southwardly they were either less common (Korab Mts, 37.1%), or completely missing, as in the Pirin Mts. In the latter mountain range (BEZ, VICH), the triploid cytotype completely predominated (94%), while tetraploids constituted a minor cytotype. The populations from the Rila Mts are very variable in cytotype composition (Table 1; Fig. 2). In *P. serbica* only diploids were found (63 plants).

Breeding system. — Castration performed in two tetraploid plants of *P. alpicola* s.str. revealed an apomictic seed formation with 75.6% of ripe achenes from four capitula. An apomictic mode of reproduction in this taxon was further confirmed by FCSS in six other tetraploid plants from Switzerland (SIM1, MOR) and two pentaploid plants from Italy (PUF) (Table 3; Fig. 4). In both cases the ratio of DAPI fluorescence intensity of endosperm and embryo nuclei was 2.0.

In contrast, FCSS of 16 maternal plants of *P. rhodopea* (GRA-W) of three different ploidies (2*x*, 3*x*, 4*x*) showed that all plants reproduced sexually (Table 3; Fig. 4). The DAPI fluorescence intensity ratio of the endosperm and embryo was in the range of 1.33–1.75, depending on the ploidy of gametes involved in fecundation. Three analysed triploid plants produced exclusively tetraploid embryos that arose by fusion of unreduced triploid egg cells and haploid pollen (3*x* + *x*) as evidenced by presence of heptaploid endosperm (6*x* + *x*). Diploids and tetraploids usually produced the embryos of the same ploidy level as the corresponding maternal plant, but very rarely triploid embryos were also detected in both cases (Table 3). In this case, the reduced haploid ovule of the diploid mother was fertilized by diploid pollen (*x* + 2*x*), because the ploidy of endosperm was tetraploid (2*x* + 2*x*). In the tetraploid mother plant, the reduced diploid ovule was fertilized by haploid pollen (2*x* + *x*), because the endosperm was pentaploid (4*x* + *x*).

Seed progeny originating from ten diploid maternal plants of *P. serbica* was formed by the sexual pathway having diploid embryos and triploid endosperms (Table 3; Fig. 4).

Morphological variation. — The morphometric study of the *Pilosella alpicola* group revealed four morphologically separated groups corresponding to four taxa: *P. alpicola* s.str., *P. ullepitschii*, *P. rhodopea* and *P. serbica* (Fig. 5).

Cluster analyses performed on 21 populations of the *P. alpicola* group consistently proved the existence of four clusters

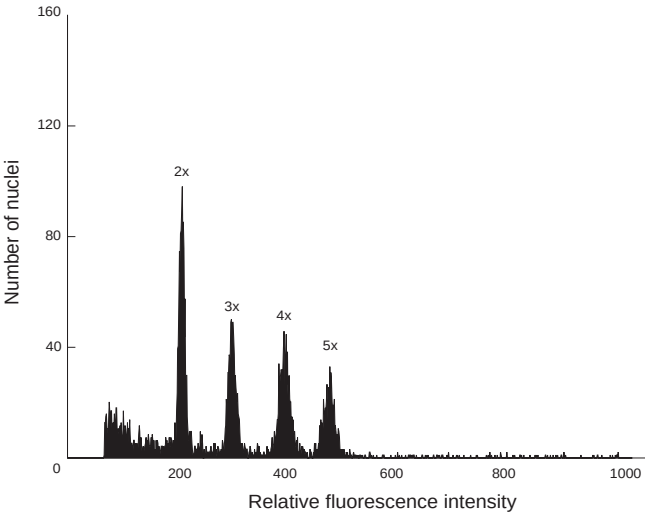

Fig. 3. Histogram of the relative DNA content of DAPI-stained nuclei in simultaneous analysis of diploid (2*x*, *P. ullepitschii*), triploid (3*x*, *P. rhodopea*), tetraploid (4*x*, *P. alpicola* s.str.) and pentaploid (5*x*, *P. alpicola* s.str.) plants of the *Pilosella alpicola* group.

Table 3. Reproduction pathways in three taxa of the *Pilosella alpicola* group detected by flow cytometric seed screen analysis.

Taxon	Locality	PLmat/N	Nseed	PLem/PLen	Fluorescence intensity endosperm/embryo ratio	Ploidy egg cell	Ploidy pollen	Embryo origin
P. alpicola s.str.	SIM1	4*x*/4	19	4*x*/8*x*	2.00	4*x*	–	Autonomous apomixis
P. alpicola s.str.	MOR	4*x*/2	3	4*x*/8*x*	2.00	4*x*	–	Autonomous apomixis
P. alpicola s.str.	PUF	5*x*/1	3	5*x*/10*x*	2.00	5*x*	–	Autonomous apomixis
P. rhodopea	GRA-W	2*x*/5	35 1	2*x*/3*x* 3*x*/4*x*	1.50 1.33	1*x* 1*x*	1*x* 2*x*	Sexual Sexual
P. rhodopea	GRA-W	3*x*/3	29	4*x*/7*x*	1.75	3*x*	1*x*	Sexual
P. rhodopea	GRA-W	4*x*/8	1 48	3*x*/5*x* 4*x*/6*x*	1.67 1.50	2*x* 2*x*	1*x* 2*x*	Sexual Sexual
P. serbica	KOP	2*x*/10	42	2*x*/3*x*	1.50	1*x*	1*x*	Sexual

Abbreviated column headers are as follows: PLmat/N, mother plant ploidy level/number of maternal plants analysed; Nseed, number of seeds; PLem/PLen, ploidy of embryo/endosperm.

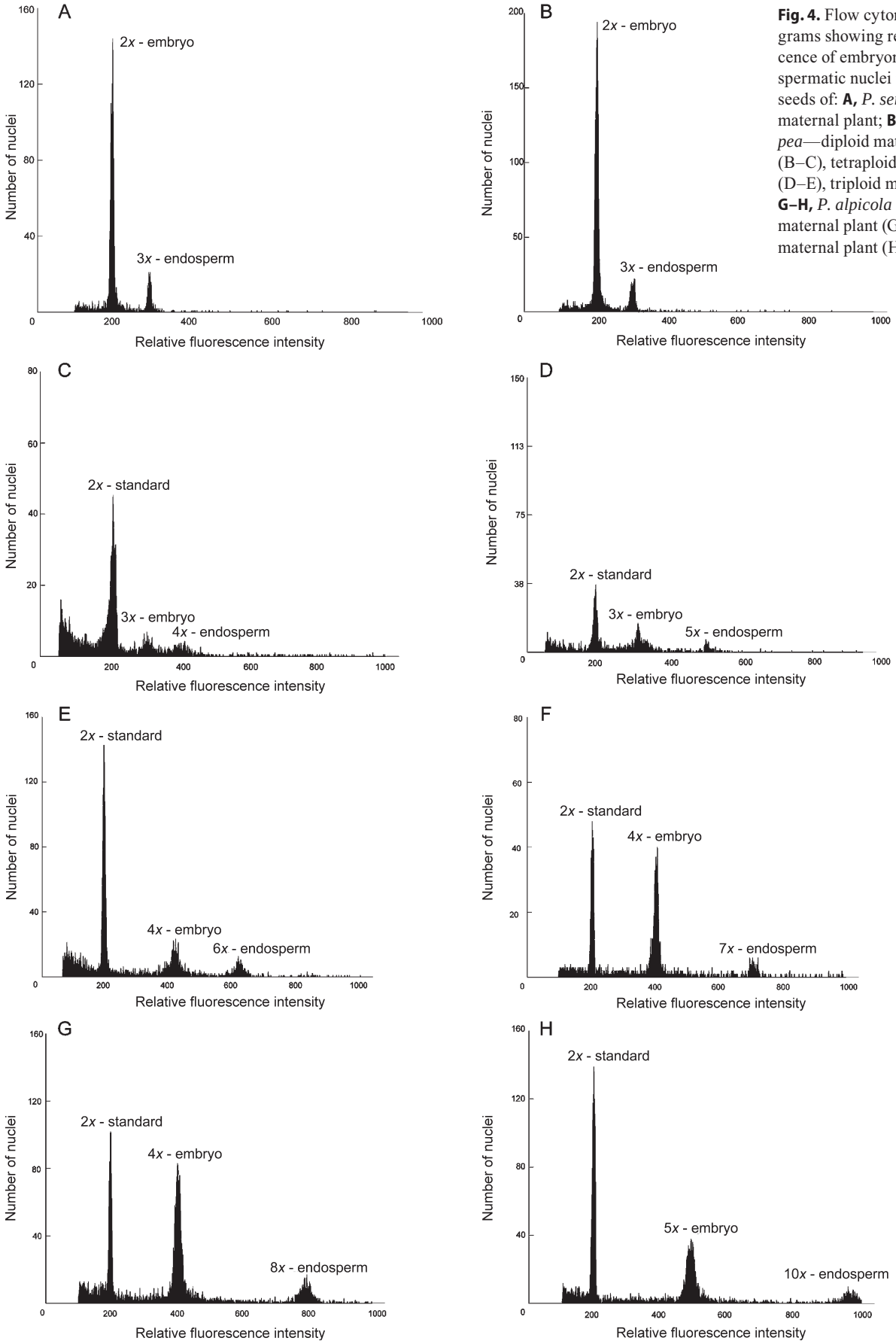

Fig. 4. Flow cytometric histograms showing relative fluorescence of embryonic and endospermatic nuclei isolated from seeds of: **A,** *P. serbica*—diploid maternal plant; **B–F,** *P. rhodopea*—diploid maternal plant (B–C), tetraploid maternal plant (D–E), triploid maternal plant (F); **G–H,** *P. alpicola* s.str.—tetraploid maternal plant (G), pentaploid maternal plant (H).

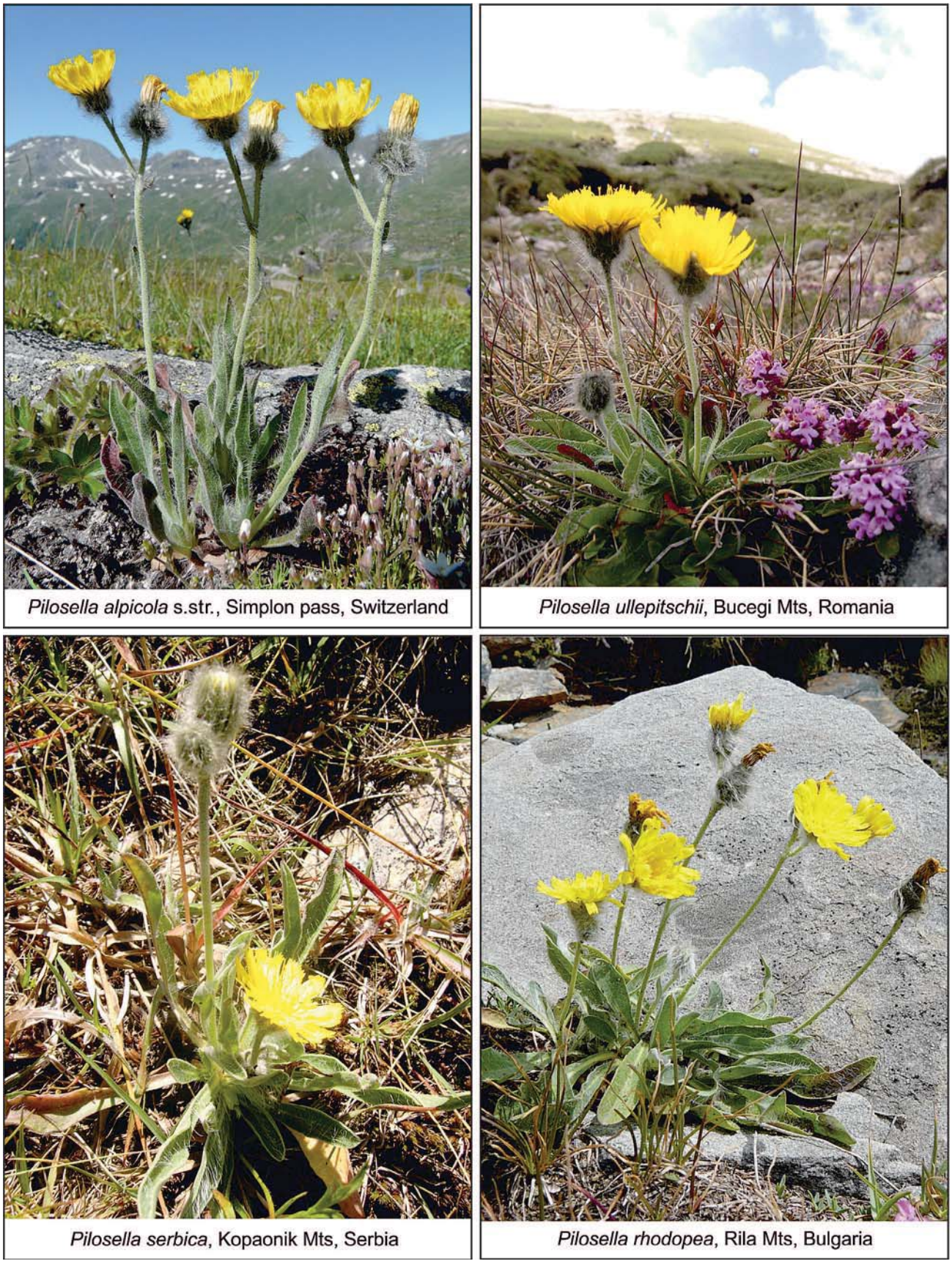

Fig. 5. Four species of the *Pilosella apicola* group. Photos by B. Šingliarová and P. Mráz.

Table 4. Total canonical structure (CDA1: can1, can2, can3) showing correlation of the characters[a] measured in the *Pilosella alpicola* group with the axes (highest values in bold).

Character	can 1	can 2	can 3
LNo	0.2472	0.0909	0.2074
LoL	–0.3120	0.4793	0.1478
LoW	0.0000	–0.0248	–0.0026
LiL	0.1046	0.4878	0.2171
LiW	–0.4113	0.1760	0.1520
LoDT	0.1616	0.5787	0.0042
LiDT	–0.1617	0.3527	0.0275
LoLT	–0.1251	**0.7225**	–0.0158
LiLT	–0.2724	0.4963	–0.0048
LoDS	0.7768	–0.0526	–0.1190
LiDS	**0.9158**	–0.0736	–0.0175
LobDS	**0.9376**	–0.0143	–0.0506
LibDS	**0.9754**	–0.0979	–0.0172
LbDG	0.1803	–0.1668	**0.7240**
LmDG	–0.0822	–0.2201	**0.8406**
LSNo	–0.3735	–0.1908	0.0714
LSL1	–0.2861	0.1733	0.3085
LSL2	–0.2586	–0.1300	0.2970
SL	–0.2370	0.5840	0.2443
SLL	–0.2403	0.0762	–0.0690
SDT	0.1804	–0.0087	0.4883
STL	–0.0244	0.3161	–0.0989
STC	–0.1644	0.4701	–0.1087
SDS	0.8341	0.0780	0.0445
SDG	–0.5554	0.4569	0.0048
CNo	0.1616	**0.7930**	0.1983
CaNo	0.0542	0.2306	**0.6325**
AL	0.1628	0.0075	0.1688
INo	–0.3090	–0.0172	0.4803
IL	–0.1060	0.0629	0.4570
IW	–0.1744	–0.1576	0.1103
ITL	–0.1834	0.0173	–0.3422
ITC	–0.2042	0.0676	0.0611
IC	**–0.9809**	–0.0925	0.0148
IDT	–0.7809	0.0004	0.0246
FNo	–0.2259	0.5760	–0.0753
FL	–0.4312	0.2979	–0.2291
FW	–0.1148	0.3824	–0.1714
StyL	–0.4848	0.3450	–0.0893
LoL/LoW	–0.1046	0.0865	0.1754
LiL/LiW	0.2897	0.1049	0.0452
FW/FL	–0.1444	0.5812	0.0438
StyL/FL	0.3138	0.2278	0.0296

[a] For character codes see Table 2.

(UPGMA plot presented here, Fig. 6). The population from the type locality of *P. serbica* (Kopaonik Mts, Serbia) was separated at the highest level of dissimilarity. The second cluster consisted of three sub-clusters. The first sub-cluster included all Carpathian populations (*P. ullepitschii*), but one—the southernmost Carpathian COZ population which fell into the second sub-cluster merging all populations from the Balkans (*P. rhodopea*). Three populations from the Alps formed the third sub-cluster (*P. alpicola* s.str.).

CDA1 (Fig. 7; Table 4) was performed to test for morphological differentiation of the *Pilosella alpicola* group and at the same time to identify morphological characters that are most suitable for distinguishing the four groups revealed by cluster analyses. The analysis shows delimitation of the individuals into four well-separated groups corresponding to four different geographical regions—the Alps, the Balkan mountains, Serbia and the Carpathians (Fig. 7). The plants from the Carpathian COZ population, however, were grouped together with the Balkan plants. The Carpathian plants without COZ population were separated along the first canonical axis and differ from the plants from other regions by less numerous stellate trichomes on leaves (LiDS, LobDS, LibDS) and dark colour involucres (IC). Along the second axis there is an isolated group of individuals from the Alps with a higher number of capitula (CNo) and longer trichomes on the outer leaves (LoLT). The last group, plants from the Kopaonik Mts, was differentiated from the remaining Balkan populations and COZ population along a third axis correlated with density of the glandular trichomes on the leaves (LmDG, LbDG) and the number of aborted capitula (CaNo).

Classificatory discriminant analysis (both parametric and non-parametric, data not shown) correctly classified plants into four groups with the highest percentage possible—100%.

Karyological and flow cytometric study revealed four different ploidy levels within *P. rhodopea*. Therefore, we performed **PCA** in order to test possible morphological differentiation between the cytotypes. The analysis showed no clear pattern correlated with ploidy level within *P. rhodopea* (Fig. 8). On the other hand, according to **PCoA** (Fig. S2), slight differentiation was found in the group of Alpine plants of *P. alpicola* s.str. Exclusively pentaploid populations from PUF (Italy) tend to be separated from the two other Swiss tetraploid populations along the second axis. This separation was caused by differences in length and width of the leaves and the plant height (LoL, LiL, LoW, LiW, SL).

We also tested the importance of quantitative and ratio characters only, excluding all semiquantitative characters. The **CDA2** differentiated the plants from the Alps from the rest along the first axis (Fig. 9). Alpine plants had deeper branched stems (ALL) and more dense simple trichomes on their leaves (LiDT, LoDT). Individuals from the Balkans and the Carpathians were partially intermingled. Carpathian plants were separated from the plants that originated from the Balkan's along a second axis due to the longer styles (StL) and flowers (FL), while plants from the Kopaonik Mts have higher numbers of capitula (CNo), involucral bracts (INo) and flowers (FNo) than plants from remaining Balkan mountain ranges.

The results of exploratory data analysis (means, standard deviation, minimum, maximum, 10% and 90% percentiles, frequencies of semiquantitative and binary characters) showed that recognised taxa differ mainly in semiquantitative characters (e.g., LoDS, LiDS, LibDG, IC; see Table 5 and Table S2). This is in accordance with the results from CDA2 suggesting that the quantitative characters alone are insufficient for correct identification of the *Pilosella alpicola* taxa.

Based on the detailed morphometric study, we provide the determination key for taxa recognised in this study (Appendix).

Variation in *rps16-trnK*, and ITS sequences. — The length of the aligned matrix of the *rps16* gene and the *rps16-trnK* intergenic spacer was 903 bp. Three sequenced plants belonging to three species had identical cpDNA sequences (GenBank accession numbers HM627315–17).

Only a limited amount of variation was recorded in nuclear ITS1-5.8S-ITS2. Therefore, we did not perform any phylogenetic analyses, but a comparison of poly-

morphic sites is provided in Table 6. The total length of the ITS1-5.8S-ITS2 region was 639 bp. We found one substitution at position 583 (ITS2) in one sample of *P. ullepitschii* exhibited C, other *P. ullepitschii* accessions showed additive polymorphism—Y (C+T, with much stronger C signal in all samples), and all remaining taxa of the group had T. *Pilosella petraea* showed at positions 74 (ITS1) and 612 (ITS2) two other substitutions—C and T respectively, both unique among all so far sequenced *Pilosella* species (Table 6; Table S1). *Pilosella cymosa* had one unique substitution among all so-far sequenced *Pilosella* taxa (including another *P. cymosa* accession) at site 559 having T instead of C. In addition, in *P. petraea* we recorded one deletion (G) in one ITS copy at position 70 and in *P. cymosa* another deletion (C) in one ITS copy at position 88. In both cases, the deletions resulted in one nucleotide shift and overlapping peaks (only sequences without deletions are given in Table 6 and Table S1).

In total, we recorded 23 different intra-individual polymorphic nucleotide sites in the *P. alpicola* group, 11 in ITS1 and 12 in ITS2. None substitution or nucleotide polymorphism was found in the highly conservative 5.8S region. Twelve of

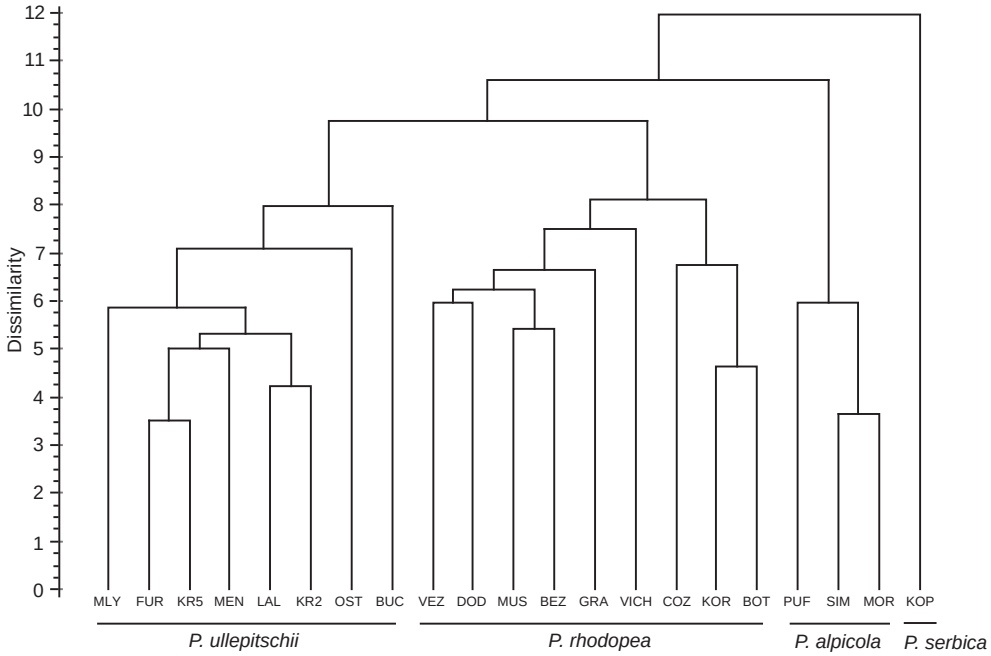

Fig. 6. Cluster analysis (UPGMA) of 21 populations of the *Pilosella alpicola* group. Population codes are given in Table 1.

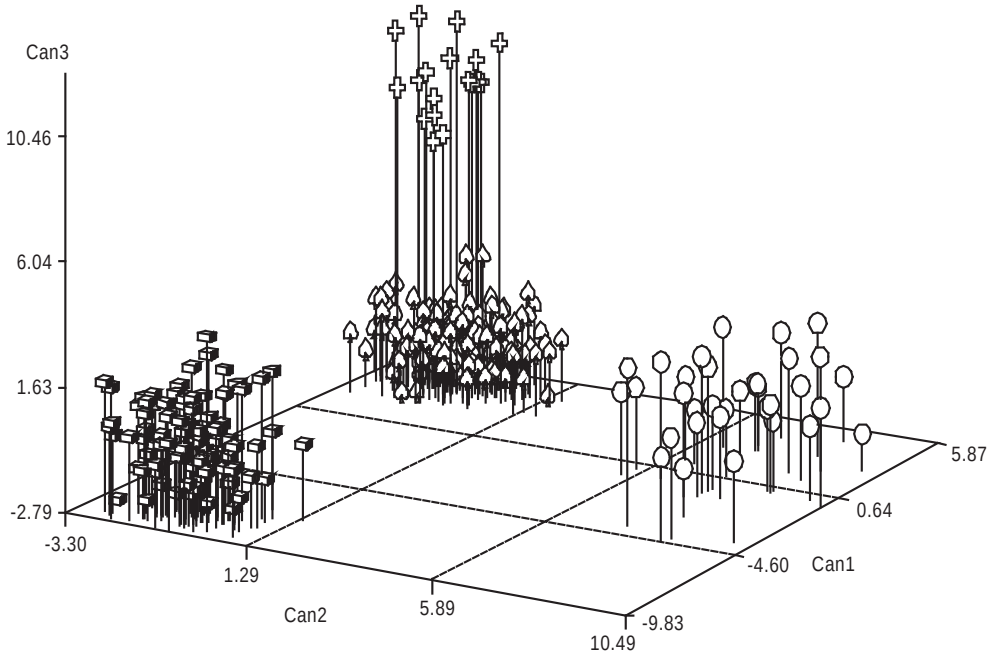

Fig. 7. Canonical discriminant analysis (CDA1) based on 43 morphological characters of individuals of the *Pilosella alpicola* group from the Alps (34 plants, circles), Carpathians (95 plants, cubes), Bulgaria, Albania, Cozia Mts in Romania (177 plants, spades) and Serbia (18 plants, crosses). The first three axes explain 83.5%, 10.2% and 6.3% of total variation, respectively.

twenty-three polymorphisms in the *P. alpicola* group were present in at least two samples and 11 were unique to individual plants. Two polymorphisms at positions 131—Y (C+T, ITS1) and 600—M (A+C, ITS2) were shared by all samples sequenced, except for one belonging to *P. petraea* and *P. cymosa*. The number of intra-individual polymorphic nucleotide sites varied between 2 to 11 per sequence (Table 6) and it was not correlated with ploidy level (Spearman rank correlation coefficient, $\rho = 0.301$, $P = 0.189$). Conversely, a significant association between this parameter and taxon was found (Kruskal-Wallis test, df = 3, $P = 0.008$; 1 accession of *P. petraea* and 1 accession of *P. cymosa* were not included in comparison). While in *P. ullepitschii*, *P. rhodopea* and *P. serbica* the number of intra-individual polymorphic sites ranged from 2 to 4 per sequence, in *P. alpicola* s.str. it was more than two-fold higher (10–11). *Pilosella petraea* had 7 and *P. cymosa* 6 polymorphic sites, each of them different from those found in the *P. alpicola* group (Table 6). Most of the intra-individual polymorphisms found in *P. ullepitschii*, *P. serbica* and *P. rhodopea* had no complementary base to other so far sequenced *Pilosella* taxa (Table 6; Table S1).

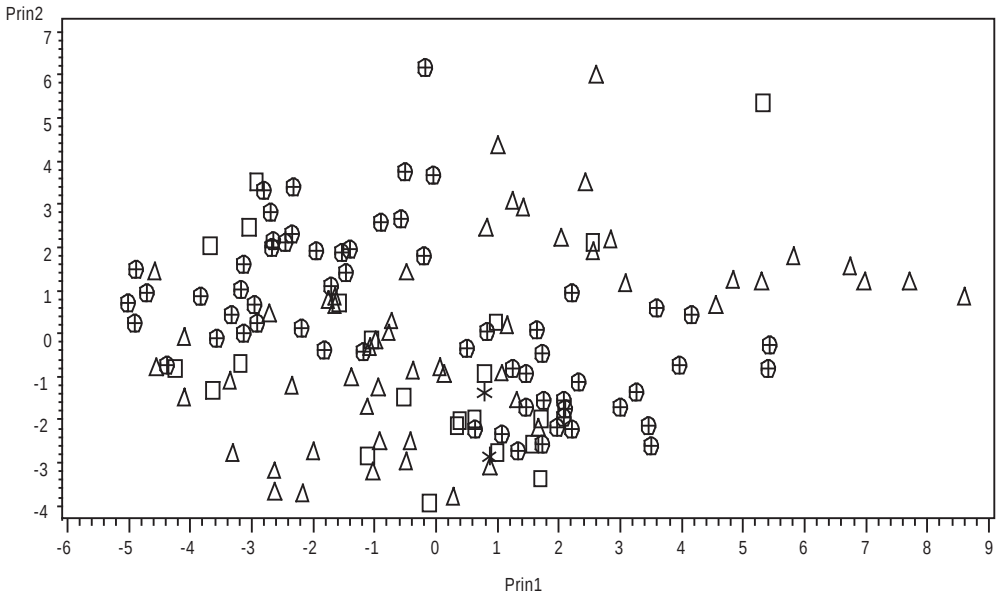

Fig. 8. Principal component analysis (PCA) based on 42 morphological characters of different cytotypes of *P. rhodopea* from Bulgaria, Albania and Cozia Mts in Romania. Diploid, circle; triploid, triangle; tetraploid, square; pentaploid, pentagon. The first two axes explain 18.9% and 10.5% of total variation, respectively.

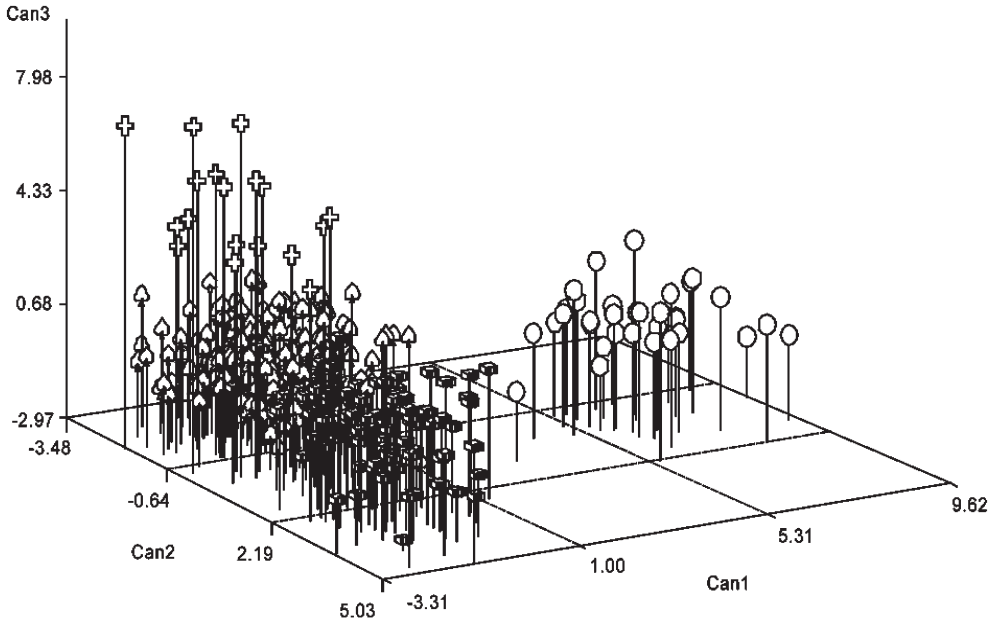

Fig. 9. Canonical discriminant analysis (CDA2) based on 26 quantitative and 4 derived ratio morphological characters of individuals of the *Pilosella alpicola* group from the Alps (34 plants, circles), Serbia (18 plants, crosses), Balkan Peninsula and Cozia Mts (177 plants, triangles) and Carpathians (95 plants, squares). The first three axes explain 56.7%, 27.1% and 16.2% of total variation, respectively.

Table 5. Frequencies (in %) of particular states of semiquantitative and binary characters[a] in different taxa of the *Pilosella alpicola* group.

	P. alpicola s.str.			*P. ullepitschii*			*P. rhodopea*			*P. serbica*		
State	0	1	2	0	1	2	0	1	2	0	1	2
LoDS	15	79	6	100	0	0	7	46	47	6	83	11
LobDS	6	85	9	100	0	0	0	21	79	0	22	78
LiDS	6	47	47	100	0	0	0	12	88	0	28	72
LibDS	12	47	41	100	0	0	0	0	100	0	0	100
LbDG	79	21	0	84	16	0	77	23	0	0	11	89
LmDG	94	6	0	68	32	0	94	6	0	0	11	89
SDG	24	76	0	53	47	0	46	47	7	0	17	83
SDT	6	32	62	19	61	20	21	60	19	39	61	0
SDS	0	18	82	27	72	1	0	10	90	0	0	100
STC	9	91	–	26	74	–	89	11	–	100	0	–
ITC	74	26	0	0	0	100	97	3	0	89	11	0
IC	24	61	15	0	0	100	47	46	7	30	70	0
IDT	0	26	74	24	60	16	44	66	0	83	17	0

[a] For character codes see Table 2.

■ DISCUSSION

Ploidy level variation, cytotype pattern and breeding system. — Including present and published karyological data, the *Pilosella alpicola* group shows a clear geographic and taxon-specific pattern (Fig. 2). *Pilosella ullepitschii* is an exclusively diploid and a strictly outcrossing Carpathian taxon (Murín & al., 1999; Šingliarová & Mráz, 2009). The only published tetraploid record from the Vysoké Tatry Mts (Uhríková & Dúbravcová, 2000) has never been confirmed in spite of a large number of individuals analysed from 13 localities (Šingliarová & Mráz, 2009) and we consider this record as questionable. Based on the analyses of the plants from the type locality (Mt. Kopaonik, Serbia), *Pilosella serbica* is also a diploid sexually reproducing species (Szeląg & al., 2007 and this paper). Further, a karyologically uninvestigated population was found in the Prokletije Mts in Montenegro (Szeląg, 2008; Šingliarová & al., in prep.).

Our results confirm that plants of *P. alpicola* s.str. in the Wallis Alps are tetraploid (cf. Favarger, 1959), but also reveal a new pentaploid cytotype. Both cytotypes have non-overlapping distributions: tetraploids occur in the Wallis Alps only, while pentaploids were ascertained only from the Italian Dolomites. Such allopatric distribution of tetra- and pentaploids suggests polytopic origin of polyploids. Tetraploid and pentaploid plants of *P. alpicola* s.str. reproduce apomictically, which is mirrored in low allozyme variation (Šingliarová & al., in press). However, the tetraploids are more variable than the almost genetically uniform pentaploids (Šingliarová & al., in press). Higher level of genetic variation found in the Wallis Alps might suggest some level of facultative sexual reproduction retaining in tetraploids. Facultative apomixis is indeed quite common in the *Pilosella* polyploids (e.g., Gadella, 1987; Pogan & Wcisło, 1995; Skalińska, 1971, 1973; Krahulcová & al., 2000, 2009a).

The Balkan subendemic *P. rhodopea* was cytologically the most variable taxon. We confirmed the existence of diploid and triploid cytotypes already reported by Šingliarová & Mráz (2009), Vladimirov & Szeląg (2001) and Krahulcová & al. (2009b). In addition, we found two new ploidies—4*x* and 5*x*. Interestingly, we found a high proportion of mixed-ploidy populations (75%) in *P. rhodopea*. A substantially lower proportion of mixed-cytotype populations was recorded in other *Pilosella* taxa studied in detail: *P. bauhini* (8.3%; Rotreklová, 2004), *P. officinarum* (12%; Mráz & al., 2008) and *P. echioides* (24%; Trávníček & al., 2011). However, proportion of ploidy-mixed populations revealed in two former studies could be influenced by lower number of analysed plants. Moreover, a significant impact of sampling strategy on cytotype composition has recently been shown (Duchoslav & al., 2010; Šafářová & Duchoslav, 2010). Here, we underline the importance of hybridization between cytotypes and participation of reduced and unreduced gametes in formation, maintenance of ploidy level variation and frequent occurrence of mixed ploidy populations in *P. rhodopea*. Furthermore, our data suggest a lack of strong reproductive barriers between cytotypes of *P. rhodopea*. Although 2*x* and 4*x* plants mostly produced offspring of identical ploidy (Table 3), rare triploid progeny were detected too. Triploids arose through hybridization between 2*x* and 4*x* (in both directions) (Table 3; Fig. 4). The triploid mother plants included in this study produced only a tetraploid progeny resulting from the fusion of an unreduced triploid ovule (formed via apomeiosis) and haploid sperm, although our unpublished data (Šingliarová & Mráz, unpub.) as well as the data from other genera (Cifuentes & al., 2010; Cosendai & Hörandl, 2010) revealed the formation of ovules with more variable ploidy level (including aneuploid one). Similar results were obtained from inter- and intraploidal experimental crosses in *P. echioides* (Peckert & Chrtek, 2006), where triploids originated mostly from 2*x* × 4*x* (both directions) and from 4*x* (mother plant) × 3*x* (pollen donor) crosses. Furthermore, if triploid mother plants of *P. echioides* were crossed with diploid pollen donors, mostly tetraploid progeny was obtained (Peckert & Chrtek, 2006), which agrees with our field data. Our results show that all studied *P. rhodopea* cytotypes are, at least to a certain level, fertile, including the triploids, which moreover substantially contribute to the formation of tetraploids, in accordance to the triploid bridge hypothesis (Harlan & de Wet, 1975).

The overall high frequency of triploid *P. rhodopea* in the Balkans (almost 50% of all plants analysed) is rather surprising as extremely low frequency of triploid embryos was detected in seed progeny analyses of 2*x*, 3*x* and 4*x* plants (Table 3). At GRA-W the cytotype proportion of adults plants was 2*x*—14.8%, 3*x*—57.4% and 4*x*—27.8% (Table 1), but the ploidy proportion in their seed progeny was quite different: 2*x*—30.7%, 3*x*—1.8% and 4*x*—67.5% (Table 3). Thus, there were less triploids in seed samples than expected from the cytotype frequency in the field. Although our results might be biased by a low number of plants used in FCSS (n = 16), Trávníček & al. (2011) observed the same shift in *P. echioides*.

Table 6. Variation in ITS region (ITS1-5.8S-ITS2) of the *Pilosella alpicola* group, *P. cymosa* and *P. petraea* sequenced in the present study and of *P. breviscapa*, *P. glacialis* (= *Hieracium angustifolium*) and *P. lactucella* based on ITS sequences by the other authors (source GenBank). Intraindividual polymorphisms are marked by grey background.

			Alignment position																																			
Taxon-accesion[a]	PL[b]	N[c]	39	46	55	70	74	75	76	100	104	114	116	118	131	206	208	224	231	445	449	451	463	479	485	494	511	522	536	537	547	557	559	562	573	583	596	600	602	612
u-MLY14-HM627304	2*x*	3	C	T	A	S	A	T	A	G	A	A	C	G	Y	T	T	G	C	T	G	G	C	C	T	T	G	T	T	T	T	C	C	C	C	C	C	M	A	G
u-OST17-HM627307	2*x*	4	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	Y	·	M	·	·
u-BAR6-HM627292, *u*-BUC7-HM627294	2*x*	3	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	Y	·	M	·	·
u-SMA19-HM627312	2*x*	4	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	Y	·	·	·	·	Y	·	M	·	·
r-VEZ11-HM627313	2*x*	4	·	·	·	G	·	·	·	·	R	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	K	·	·	·	·	·	T	·	M	·	·
r-VEZ18-HM627314	2*x*	3	·	·	·	G	·	·	·	·	R	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-COZ1-HM627295, *r*-COZ2-HM627296	2*x*	2	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-BOT3-HM627293	2*x*	3	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	Y	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-GRA12-HM627300, *r*-GRA14-HM627301, *r*-MUS7-HM627305, *s*-KOP17-HM627303	3*x* 4*x* 5*x* 2*x*	2	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-DOD14-HM627299	3*x*	4	·	·	·	G	·	·	·	·	R	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	Y	·	·	T	·	M	·	·
r-DOD7-HM627298, *s*-KOP7-HM627302	2*x*	3	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	Y	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
a-SIM3-HM627310	4*x*	11	·	·	·	G	·	·	R	·	R	M	Y	R	Y	·	Y	S	·	Y	·	·	·	·	·	T	·	·	·	·	·	·	·	Y	·	T	·	M	·	·
a-SIM4-HM627311	4*x*	11	·	Y	R	G	·	·	·	·	R	M	Y	R	Y	·	Y	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	Y	T	·	M	R	·
a-SCH13-HM627309, *a*-PUF6-HM627308	5*x*	10	·	Y	R	G	·	·	·	·	R	M	Y	R	Y	·	·	·	·	Y	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	R	·
p-O1-HM627306	2*x*	7	Y	·	·	G	C	·	·	·	·	·	·	·	T	·	·	·	Y	·	K	·	Y	·	·	T	R	Y	·	·	·	·	·	·	·	T	Y	A	·	T
c-CYM1-HM627297	2*x*	6	·	·	·	G	·	·	·	R	·	·	·	·	T	Y	·	·	·	·	·	R	·	Y	·	T	·	·	Y	·	·	Y	T	·	·	T	·	A	·	·
H. breviscapum- AJ633393			·	C	G	G	·	G	·	·	·	C	T	A	T	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	A	G	·
H. angustifolium- AJ633407			·	C	G	G	·	·	·	·	·	C	T	A	T	·	·	·	·	C	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	A	G	·
H. lactucella- AJ633389			·	C	G	G	·	·	·	·	·	C	T	A	T	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	A	G	·

[a] Initial letter in sample code indicates species studied: *a*, *P. alpicola* s.str.; *c*, *P. cymosa*; *p*, *P. petraea*; *r*, *P. rhodopea*; *s*, *P. serbica*; *u*, *P. ullepitschii*. For population codes see Table 1.
[b] PL, ploidy level.
[c] N, total number of intraindividual polymorphisms per accession.

It seems that the predominant triploids in *P. rhodopea* may have some advantage over other cytotypes. However, the underlying mechanisms of selection for triploid plants are not yet known and deserve further experimental approach to infer the differences and/or patterns in intercytotype pollen competition, seed set, seed dispersal, germination rate, seedling survival, tolerance to competition, etc. Since spatial and habitat differentiation might also play a role in maintaining the high frequency of triploids, a detailed study of populations with mixed cytotypes might shed more light on cytotype coexistence and frequency at microspatial scales. For instance, only tri- and tetraploid plants were found on intensively grazed pastures in the GRA population, while on the ridge situated westerly (GRA-W) besides 3*x* and 4*x* also 2*x* plants were recorded. Because the latter locality was less affected by grazing and because there were also naturally disturbed, competition-free sites (scree), this finding may suggest niche differentiation among cytotypes (see Felber-Girard & al., 1996; Hardy & al., 2000; Schönswetter & al., 2007a; Raabová & al., 2008) and lower competitive ability of diploid cytotype in dense vegetation.

Sympatric cytotype pattern with predominating mixed-ploidy populations and no obvious reproductive barriers between the *P. rhodopea* cytotypes strongly suggest a primary origin of polyploids within diploid populations. This suggestion is further supported by morphometric analysis from a recent study and from allozyme pattern (Šingliarová & al., in press) indicating no phenetic and genetic differentiation between diploids and polyploids. Of course, we cannot exclude the possibility that *P. rhodopea* polyploids might be the immigrants into diploid populations and that frequent hybridization between cytotypes could completely blur original genetic differences. Primary polyploid formation in diploid populations of *P. echioides* was suggested already by Peckert & Chrtek (2006). Both *P. alpicola* and *P. echioides* seem to be very rare documented cases of in situ origin of polyploids, as Petit & al. (1999) considered that most (if not all) of so-far studied contact zones of diploid and polyploid cytotypes are of secondary origin.

Morphological differentiation. — Morphometric study of the *Pilosella alpicola* group revealed the existence of four well-separated groups of plants corresponding to four taxa—*P. alpicola* s.str., *P. rhodopea, P. ullepitschii* and *P. serbica* (Fig. 5). They differ mainly in semiquantitative traits like density, length and colour of indumentum on the leaves, stems and inflorescences. These characters are considered to be involved in evapotranspiration and water-stress adaptation in plants (e.g., Ehleringer, 1982; Sandquist & Ehleringer, 1997; Ackerly & al., 2000). The density of stellate trichomes is higher and simple trichomes are brighter in *P. rhodopea* and *P. serbica*, which originate from southern ranges, than in *P. ullepitschii* occurring in more northern latitudes with more than 40% higher precipitation during the growing season in comparison to southern latitudes. These morphological differences are stable in cultivation and thus suggest a genetic basis of such variation. Interestingly, phenotypic differentiation indicating an adaptation to different moisture conditions is strongly supported also by ecophysiological traits involved in water-regime, like water-use efficiency (measured through carbon isotope discrimination—$\Delta^{13}C$), specific leaf area and percentage of leaf carbon. All these data likely suggest an adaptive evolution in allopatry of Carpathian and Balkan taxa (Mráz & al., in prep).

The results of morphometric analyses confirm the already suggested conspecific status of *Hieracium alpicola* subsp. *micromegas* with *Pilosella rhodopea* and *P. alpicola* subsp. *furcotae* with *P. ullepitschii* (cf. Szeląg, 2008). When analysed separately, slight morphological differences were observed between vicariant Alpine tetra- and pentaploids of *P. alpicola* s.str. (PCoA, Fig. S2). This differentiation might be the result of either a polytopic origin and/or long-term vicariancy. Multiple origins of *P. alpicola* s.str. are supported not only by cytogeographic and ITS patterns revealed in this study, but also by small differences in allozyme profiles (Šingliarová & al., in press). Indeed, polytopic formation of polyploid lineages is a common phenomenon in vascular plants (e.g., Soltis & Soltis, 1999), and it was suggested also for the polyploid *Pilosella officinarum* complex on the basis of cytogeographic pattern (Mráz & al., 2008).

ITS variation and origin of polyploid taxa. — The whole *Pilosella alpicola* group shows very low interspecific ITS variation and complete uniformity in plastid DNA sequences. This is in concordance with overall phylogenetic pattern observed in the genus *Pilosella* (Fehrer & al., 2007a) suggesting rather young age of this genus. Our nrDNA data confirmed the close relationships between the members of the *Pilosella alpicola* group, with exception of the nominate species, *P. alpicola* s.str. In spite of very limited interspecific variation, our analyses revealed frequent intra-individual ITS polymorphism. In apomictic *P. alpicola* s.str. this polymorphism can be partially explained by its allopolyploid origin and apomictic reproduction. Both phenomena could substantially retard or even completely suppress homogenisation processes of different ITS repeats found in many plant taxa (cf. Sang & al., 1995; Wendel & al., 1995; Aguilar & al., 1999) due to reduced/lacking recombination rate and meiotic paring in the case of non-homologous ITS regions (cf. Campbell & al., 1997; Fehrer & al., 2009; Hörandl & al., 2009; Záveská-Drábková & al., 2009). However, most of the intra-individual polymorphic sites found in the *P. alpicola* group (excluding *P. alpicola* s.str.) do not show any additive pattern, and therefore they cannot be interpreted by recent introgression from other taxa that have been sequenced so far (Table 6; Table S1). It means that standing intra-individual ITS variation might be due to the past hybridization events involving already extinct lineages, as it was suggested for the closely related genus *Hieracium* (Fehrer & al., 2009), and/or due to very high mutation and slow homogenisation rates. At least two nucleotide polymorphisms at positions 131 and 600 were found in all accessions irrespective of their taxonomic, geographic and ploidy level status (Table 6). This fact suggests that these polymorphisms had to precede the diversification and polyploidization events of the *Pilosella alpicola* group. Similar pattern was observed in diploid and polyploid *Hieracium* taxa (Fehrer & al., 2009).

The high number of polymorphic nucleotide sites found in *P. alpicola* s.str. by direct sequencing indicates the existence of at least two divergent ITS copies, and thus suggests its

allopolyploid origin. Based on morphological, molecular and ecological data we hypothesize that *P. alpicola* s.str. is a hybridogeneous species between *P. glacialis* (Reyn. ex Lachen.) F.W. Schultz & Sch.Bip. (syn. *Hieracium angustifolium* Schur.) (or some closely related so far not sequenced taxon) from the Alps and *P. rhodopea* from the Balkans. In total, the ITS sequences of *P. rhodopea* (and *P. serbica*) and *P. glacialis* differed in eight nucleotide positions, and in seven of them we recorded an additive pattern in *P. alpicola* s.str. More important, at position 445 three from four sequenced accessions of *P. alpicola* s.str. had Y (C+T), while the other samples of *P. alpicola* s.l., including all remaining *Pilosella* species had T, but *P. glacialis* having C. This suggests that the second parental species could be *P. glacialis* (Table 6). The complementary characters of ITS copies, however, were not consistent in all sequenced samples of *P. alpicola* s.str. (Table 6). Interestingly, all sequenced individuals of *P. alpicola* s.str. showed at position 104 R (A+G), similar to two diploid (VEZ) and one triploid plant (DOD) of *P. rhodopea* (Table 6). This polymorphism was not detected in all remaining samples exhibiting adenin at the same position, like other *Pilosella* taxa. This likely suggests that the second putative parent might be *P. rhodopea*. *Pilosella alpicola* s.str. shows some intermediate morphological characters which might originate from introgression with *P. glacialis* (higher number of capitula, longer acladia and taller stems). Nevertheless, *P. alpicola* s.str. is still morphologically closer to *P. rhodopea* than to *P. glacialis*. This might be due to different levels of genomic dosage of both putative parental taxa, with greater influence of *P. rhodopea*. Although the ratio of divergent ITS copies should be estimated by rigorous quantitative methods, the higher signal of nucleotides originated from *P. rhodopea* at all polymorphic sites might indeed suggest a larger amount of *P. rhodopea* genome in *P. alpicola* s.str. *Pilosella glacialis* is, like *P. alpicola* s.str., an endemic species of the Alps with similar ecological demands. Although the current range of *P. rhodopea* does not overlap with those of *P. glacialis*, both taxa could have hybridized in the past, either in periglacial Alpine refugia or in the area between the Alps and the Balkan mountains. We hypothesize that the first putative parent subsequently went extinct and left only his progeny—*P. alpicola* s.str.

It is not known if apomictic reproduction of *P. alpicola* s.str. arose as a consequence of interspecific hybridization between two divergent taxa reproducing sexually (cf. Asker & Jerling, 1992; Carman, 2001), or if the hybridization already involved at least one parental species with apomictic reproduction. Few studies have reported *P. glacialis* as a diploid species (Favarger, 1969a,b), and as a rule in the genus *Pilosella* the diploid taxa reproduce exclusively sexually (Krahulcová & al., 2000). Because all tested cytotypes of *P. rhodopea* are sexual too, the first hypothesis might be correct. However, we have only limited information on cytotype variation in *P. glacialis* (see above) and therefore we cannot exclude the possibility that some polyploid apomictic cytotypes might be found in this taxon.

In contrast to *P. alpicola* s.str., our morphological, cytogeographical and ITS data strongly support an autopolyploid origin of *P. rhodopea* polyploids. Moreover, sharing of the same allele suite and presence of both balanced and unbalanced heterozygotes in *P. rhodopea* are also consistent with this assumption (Šingliarová & al., in press).

Biogeographic pattern. — The modern range of the *Pilosella alpicola* group is restricted to disjunct mountain ranges (Fig. 2). However, during long glacial periods one might expect that the *P. alpicola* group had a more continuous distribution because the ice ages provided more favourable conditions for many alpine and mountain taxa than the short and warm interglacial periods (e.g., Schmitt 2007). During cold periods alpine and mountain taxa might expand their ranges and overcome potential dispersal barriers (e.g., lowlands, large river valleys, etc.) that separate their modern ranges. Because our molecular data indicate a monophyletic origin of the *P. alpicola* group (with the exception of *P. alpicola* s.str.), we assume that the progenitor of this group, either an extinct or extant species, occupied a larger and more continuous range than the group does currently. Unfavourable warm climatic conditions during the Pleistocene have subsequently induced range fragmentation leading to the formation of new, morphologically distinct taxa adapted to local conditions. Thus, we suggest that the range expansion and contraction played a significant role in shaping the modern distributional and evolutionary pattern in the group.

In this way we could explain the hybridogenous origin of *P. alpicola* s.str., an endemic to the Alps. We suppose that under favourable conditions *P. rhodopea*, one of its putative parental species, underwent range expansion from its core area in the Balkans, with subsequent hybridization to *P. glacialis* (or a closely related taxon) in the Alpine periglacial refugium/refugia. Indeed, molecular and distributional patterns of animal and plant taxa support the biogeographic link between the Alps and the Balkans (e.g., Szeląg, 2006; Schmitt, 2009; Stevanović & al., 2009). Furthermore, the existence of two isolated populations of *P. rhodopea* in the Southern Carpathians provide further evidence for former broader distribution of *P. rhodopea*, and underline floristic and faunistic affinities between the Southern Carpathians and the Balkans (e.g., Szeląg, 2006; Schmitt, 2009).

In spite of an efficient mechanism for colonisation and long-distance seed dispersal via achenes with pappus, the modern range of the *P. alpicola* group is very restricted. This might suggest a high extinction rate either during or more likely after range expansions. Generally, the *Pilosella alpicola* taxa are week competitors, and in comparison to other *Pilosella* taxa, vegetative reproduction via stolons is highly restricted. We suggest that the altered environment during unfavourably warm and humid periods (e.g., Atlantic period) led to the considerable loss of suitable habitats. Furthermore, given the weak competitive ability of *Pilosella alpicola* taxa, this may also have led to decreasing of population sizes.

Interestingly, an apomictic mode of reproduction which is considered to be very advantageous for colonising new habitats (Vandel, 1928; Bierzychudek, 1985; Asker & Jerling, 1992; Hörandl, 2006), has not contributed to the range expansion of *P. alpicola* s.str. as was observed in many apomictic taxa (e.g.,

Antennaria rosea complex: Bayer, 1990; *Paspalum simplex*: Urbani, 2002; *Taraxacum* and *Chondrilla*: Van Dijk, 2003; *Townsendia hookeri*: Thompson & Whitton, 2006; *Pilosella officinarum*: Mráz & al., 2008; *Hieracium alpinum*: Mráz & al., 2009; *Ranunculus kuepferi*: Cosendai & Hörandl, 2010). This might suggest either the existence of some effective selection mechanisms that prevent the range expansion of *P. alpicola* s.str., at least in its Swiss range (in Italian Dolomites the spreading is strongly limited due to unsuitable geological and soil conditions; Šingliarová & al., in prep.), and/or the taxon might be too young and has not had sufficient time for further spread. A similar pattern was observed in hybridogenous and agamospermous species *Erigeron trifidus*, which forms only restricted and disjunct populations (Burke & Bain, 2008).

Taxonomic position of *Pilosella petraea*. — Unfortunately we did not have suitable samples of *P. petraea* for morphometric studies due to missing data for flower traits. Nevertheless, literature data (Heuffel, 1853; Zahn, 1922–1930; Nyárády, 1965; Szeląg, 2008) as well as our measurements of leaf traits of five cultivated plants from the type locality (Šingliarová, unpub.) indicate strong morphological differentiation of this taxon from the remaining taxa of the *Pilosella alpicola* group. First, leaves were without small glandular trichomes that are characteristic of all other *P. alpicola* taxa. Furthermore, leaves had substantially fewer dense stellate trichomes on both leaf sides (with exception of leaf margins) when compared to *P. alpicola* s.l. Moreover, *P. petraea* had thicker [0.08(–0.09) mm] and setaceous-like simple eglandular trichomes, while the hairs in *P. alpicola* s.l. were thinner [(0.03–)0.04–0.05(–0.06) mm] (Šingliarová, unpub.). *Pilosella petraea* also had a higher number of capitula in synflorescence (2–15) that were smaller in size (6–8 mm, cf. Zahn, 1922–1930) than *P. alpicola* taxa having typically 1–3 larger flower heads [(6–)7–11(–13)mm] (Zahn, 1922–1930; Šingliarová, unpub.). Moreover, *Pilosella petraea* substantially differs from other taxa by its ecological demands. It grows in the crevices of calcareous rocks in mountain belt, while the representatives of the *Pilosella alpicola* group typically occur in subalpine/alpine belt on acid bedrock. ITS pattern of *P. petraea* likely suggests different evolutionary origin of this species and alongside with morphological and ecological data supports exclusion of this taxon from the *Pilosella alpicola* group. The high number of intraindividual ITS polymorphisms found in this diploid species might indicate its hybridogenous origin. Zahn (1922–1930) already pointed out that *P. petraea* is a taxon morphologically between *P. alpicola* s.l. and *P. cymosa*, suggesting its hybrid history. The ITS pattern obtained in this study (Table 6), however, does not support the hypothesis on this parental combination, although the limited number of *P. cymosa* accessions should be taken into consideration.

■ CONCLUSIONS

Our combined methodological approach enabled us to provide a new circumscription of the *P. alpicola* group and revealed auto- and allopolyploidization events operating within it. The group consists of four morphologically well-differentiated and geographically vicariant species with contrasting cytotype patterns. Interestingly, the mode of reproduction of polyploid cytotypes reflects their origin: *P. rhodopea* autopolyploids reproduce strictly sexually, while allopolyploid cytotypes of *P. alpicola* s.str. reproduce apomictically. Our data also revealed a rare primary contact zone between diploid and polyploid cytotypes of *P. rhodopea*. In spite of clear morphological separation the molecular data suggest recent diversification of the group. We hypothesize that fragmentation of more continuous ranges, range shifts connected with interspecific hybridization (as in the case *P. alpicola* s.str.) and adaptation to different climates were the main drivers of speciation in the group. Our data suggest that the *Pilosella alpicola* group is a promising model for studying plant speciation, adaptation and recent polyploidization.

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Appendix. Identification key for the *Pilosella alpicola* group.

Characters (values) are expressed as (minimum–)10th percentile–90th percentile(–maximum).
Pale: white, ivory, yellowish, silver grey.
Dark: smoky grey, grey-black, brown-grey.

1. Plant (8–)10–24(–29) cm high; acladium if present (1.0–)2.2–5.0(–5.5) cm long; simple hairs on leaves and peduncle dense; simple hairs on involucrum usually pale with dark base or occasionaly dark up to the middle. – Inflorescences (1–)2–3 (Alps: Switzerland, Italy) . . . . . ***P. alpicola* s.str. F.W. Schultz & Sch. Bip.**

1. Plant (2–)5–14(–19) cm high; acladium if present (0.1–)0.3–2.5(–3.5) cm long; simple hairs on leaves and peduncle sparse; simple hairs on involucrum pale occasionally with dark base or dark up to the middle or completely dark **2**

2. Simple hairs on involucrum consistently dark; stellate hairs on leaves rare; on peduncle sparse. – Inflorescences 1–2(–3); acladium if present (0.2–)0.3–1.0(–1.2) cm long (Carpathians: Slovakia, Poland, Romania) . . . . . ***P. ullepitschii* (Błocki) Szeląg**

2. Simple hairs on involucrum pale, occasionaly with dark base or dark up to the middle; stellate hairs on leaves and peduncle dense **3**

3. Glandular hairs on leaves and peduncle rare to sparse, inflorescences 1–2(–3); acladium if present (0.1–)0.3–3.4(–3.5) cm long (Carpathians: Romania; Balkans: Bulgaria, Albania, Macedonia, Greece) . . . . . ***P. rhodopea* (Griseb.) Szeląg**

3. Glandular hairs on leaves and peduncle dense, inflorescences 2–4; acladium (0.3–)0.4–1.5(–1.9) cm long (Balkans: Serbia, Montenegro) . . . . . ***P. serbica* (F.W. Schultz & Sch. Bip.) Szeląg**