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Genetic variation in agamospermous taxa of *Hieracium* sect. *Alpina* (Compositae) in the Tatry Mts. (Slovakia)

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Abstract. The mode of reproduction, pollen production, chromosome numbers, genetic variation (RAPD, allozymes) and overall similarity were studied in 6 species of *Hieracium* sect. *Alpina* in the Tatry Mts. (the Western Carpathians, Slovakia). All species were confirmed to be agamospermous and, except of *H. krivanense* and *H. slovacum*, lacking pollen grains. For the first time, a chromosome number is reported for *H. krivanense* ($2n = 4x = 36$). Considerable genetic variation was revealed in *H. alpinum* and a correlation between geographic and genetic distances was found in this species. Between-population variation in RAPD and allozyme phenotypes was found in *H. pinetophilum* and *H. crassipedipilum*. In all other species, allozyme and RAPD variation was low or absent. With few exceptions, the species differ in their allozyme as well as RAPD patterns. The relatedness of one population of endemic *H. slovacum* and *H. halleri* was confirmed. It is shown, that Carpathian species of the *H. fritzei* group are derived from at least two ancestors.

Key words: Compositae, *Hieracium* sect. *Alpina*, apomixis, genetic variation, RAPD, allozymes.

The evolutionary potential of a population or species largely depends on the amount and pattern of genetic variation. This variation is affected by effective population size, selection pressure, migration, population structure and mode of reproduction. In plants, many species reproduce both sexually and asexually. Asexual reproduction includes both the formation of stolons and rhizomes and apomixis (asexual seed production). Whether apomicts have a lower evolutionary potential in comparison with sexual relatives has been a debate for decades (Darlington 1939, Clausen 1954, recently van Baarlen et al. 2000). Because apomicts are clonal lineages and do not reproduce sexually, genetic variation is generated only through somatic mutation. Thus, whether they generate genetic variation sufficient for further evolution or they are simply dead ends of the evolutionary process remains puzzling (Asker and Jerling 1992). Variation within populations of apomictic species has been detected, even among offspring of apparently obligate agamospermous individuals, e.g. in many *Taraxacum* Weber species (Richards 1996). The presence of substantial levels of genetic variation has been reported among some microspecies of *Hieracium* sect. *Alpina* (Fr.) F.N. Williams in the British Isles [Stace et al. (1997) and Shi et al. (1996)].

The genus *Hieracium* L. (common name hawkweed) in the narrow sense (*Hieracium*

subgen. *Hieracium*, Sell 1987), contains herbs distributed mainly in temperate regions of the northern hemisphere (Zahn 1921–1923, Sell and West 1976). The genus is known for its taxonomic complexity, which is associated with variation in ploidy levels and breeding systems. Asexual reproduction through the parthenogenetic development of the unreduced egg cell (Antennaria-type diplospory) prevails in this genus. Sexual reproduction is rare and restricted to a few diploid ($2n = 18$) species (Schuhwerk 1996). It is generally thought, that many recent *Hieracium* agamospermous taxa sharing morphologically intermediate positions between two or more species are the results of past hybridization events (Zahn 1921–1923). However, if this is true, most of the putative parental species must be extinct and their genomes are represented only by agamospermous populations.

The European mountains provide an ideal system to investigate the processes of hawkweed microevolution. The members of sect. *Alpina* are restricted to the highest vegetation belts. These suitable habitats are separated by large areas of unsuitable lowland habitats. The scattered patches of alpine hawkweed stands resemble an archipelago. Particular mountain "islands" in Central Europe differ remarkably in the present coverage in subalpine and alpine regions, in the changes of vegetation cover during the Late Glacial Period and Holocene, as well as in the time of isolation from other treeless sites.

In the present study, we focus on two taxonomical groups within *Hieracium*: the *H. alpinum*- and *H. fritzei*-groups of species (Table 1). A narrow species concept was adopted here. For a detailed taxonomic account of these groups of species in the Western Carpathians see Chrtek (1997), Chrtek and Marhold (1998). Species of the *H. fritzei*-group differ from those of the *H. alpinum*-group by having more stem leaves which are sometimes semiamplexicaul. They are endemic to either the Sudeten Mts. (N Czech Republic, SW Poland) or the Western Carpathians. The *H. fritzei*-group occupies a morphologically intermediate position between the *H. alpinum*- and the *H. prenanthoides*-groups but it is morphologically more similar to *H. alpinum* (Chrtek and Marhold 1998). It is unknown whether the *H. fritzei*-group is monophyletic or whether particular species of this group have originated repeatedly

Table 1. Species of the *H. alpinum*- and *H. fritzei*- groups in the Tatry Mountains (the Western Carpathians). **Sex** – sexual, **apo** – apomict (agamosperm), data firstly published in the present paper are in bold

Species	2n, mode of reproduction	Total distribution area
H. alpinum L. s.str.	18, sex (Eastern Carpathians only, Chrtek 1997) 27, apo (Chrtek 1997) 36, ? (Polish Tatra Mts., Szeląg and Jankun 1997)	arcto-alpine
H. halleri Vill. (*H. alpinum* group)	27, apo (Chrtek 1997)	Alps, Western Carpathians
H. crassipedipilum (Pawl. & Zahn) Chrtek jun. (*H. fritzei* group)	**27, apo** 36, apo (Chrtek and Marhold 1998)	Western Carpathians
H. pinetophilum (Degen & Zahn) Chrtek jun. (*H. fritzei* group)	27 (Chrtek and Marhold 1998), **apo**	Western Carpathians
H. slovacum Chrtek jun. (*H. fritzei* group)	36 (Chrtek and Marhold 1998), **apo**	Western Carpathians (Belanské Tatry Mts.)
H. krivanense (Wol. & Zahn) Schljakov (*H. fritzei* group)	**36, apo**	Western Carpathians

through polytopic hybridization events between different taxa of the *H. alpinum* group and species with more leafy stems and amplexicaul leaves. Because most of the current representatives of the putative parental species are agamospermous, these hypotheses cannot be tested through experimental crossing studies.

We use two methodological approaches to identify genetic similarities and differences among populations and species. Random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990, Welsh and McClelland 1990) often reveal large amount of variation at both the population and species levels. Despite several limitations – lower reproducibility, high sensitivity to little changes in reaction conditions and dominant nature of the markers (Lynch and Milligan 1994) – RAPD analysis have been very useful for evaluating genetic variation in apomictic taxa (Bartish et al. 2001, Palacios and Gonzales-Candelas 1997). Allozyme polymorphism has also provided valuable information bearing on the reproductive behaviour, population structure and clonal diversity of agamospermous species (Hughes and Richards 1988). The main advantage of allozyme markers is their co-dominant nature but their main drawback is the limited set of marker loci available.

Our aims are the following: (i) to ascertain the mode of reproduction in the taxa of the *H. fritzei*-group, (ii) to evaluate among- and within-population variation in 6 species of the *H. alpinum*- and *H. fritzei*-groups in the Tatry Mountains, and (iii) to examine overall relationships among species of the *H. alpinum*- and *H. fritzei*-groups in the Tatry Mts.

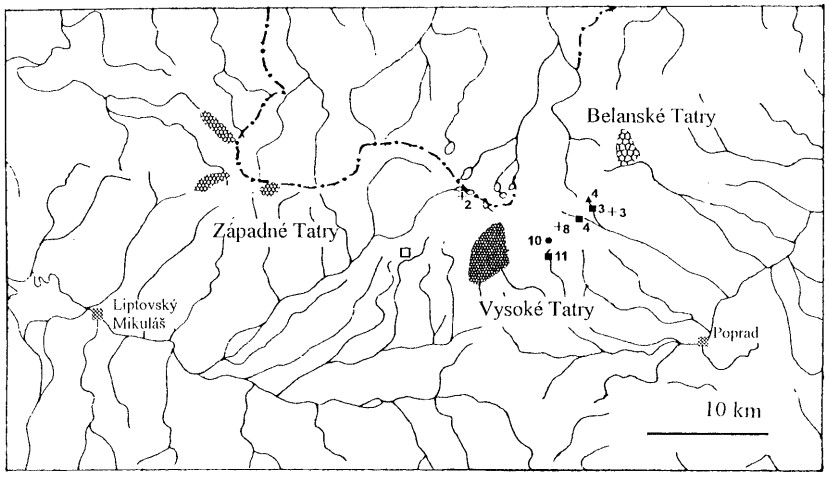

Fig. 1. Map of the Tatry Mts. with geographic locations of population samples of *H. alpinum* (▲), *H. halleri* (+), *H. crassipedipilum* (●) and *H. pinetophilum* (■). Numbers correspond to those in App 1. Where the spatial pattern of localities did not allow this kind of presentation (more localities within a small area) individual dots are substituted by textures (abbreviations follow App. 1): ▒ (Západné Tatry Mts.) Alp 1, Alp 2, Alp 3, Alp 6, Alp 8, Cras 1, Cras 2, Pin 1, Pin 6, Kriv 1, Hal 1, Hal 4, Hal 6, Hal 7; ▓ (Vysoké Tatry Mts.) Alp 5, Cras 4, Cras 5, Cras 6, Cras 7, Cras 11, Pin 5, Pin 7, Pin 8, Pin 9, Kriv 2, Hal 5, Hal 9; ▒ (Belanské Tatry Mts.) Alp 7, Cras 3, Cras 8, Pin 2, Pin 10, Slov 1; □ Cras 9, Kriv 3

Materials and methods

Study area. The Tatry Mountains (highest peak Mt. Gerlachovský štít, 2655 m) constitute the highest part of the Carpathians and are situated astride the boundary between Slovakia and Poland. In the present study, we recognize three ranges within the Slovakian Tatry mountains, a phytogeographical division proposed by Futák (1984): 1) the Západné Tatry Mts., 2) the Vysoké Tatry Mts., and 3) the Belanské Tatry Mts. (see Fig. 1). All studied species occur in the subalpine and alpine belts in grassland communities and in open-canopy dwarf pine stands.

Plants. Six species (43 populations) were studied, i.e. *Hieracium alpinum* L. s.str., *H. halleri* Vill., *H. crassipedipilum* (Pawl. & Zahn) Chrtek jun., *H. pinetophilum* (Degen & Zahn), *H. krivanense* (Wol. & Zahn) Schljakov, and *H. slovacum* Chrtek jun. (Table 1, Appendix 1). A population is considered here as the set of plants belonging to the same species and growing at the same locality. The plants were collected in the Tatry Mts. in 1996–1998 and transferred to an experimental garden in Průhonice. In some cases, leaves for DNA analysis were collected directly in the field and kept in saturated NaCl/CTAB solution until DNA was extracted in the laboratory. Voucher specimens of all plants analyzed are deposited in the herbarium of the Institute of Botany, Průhonice (PRA).

Cytology. Chromosome counts were made for all cultivated plants. Root tip cuttings of mature plants were used for karyological studies. The material was pretreated with a saturated solution of p-dichlorobenzene, fixed in a mixture of ethanol and acetic acid (3:1) and stored in 70% ethanol. The squash method and staining by lacto-propionic orceine were used (Dyer 1963).

Pollen production and mode of reproduction. To assess the presence or absence of pollen production, three or four immature florets per plant were dissected. Presence/absence of pollen was proven to be invariable within species and therefore results for each individual plant are not reported. Pollen production was ascertained for all plants involved in the present study (i.e. in cultivated plants as well as in voucher specimens of plants from which leaves were taken directly in the field).

The mode of reproduction was tested in at least 3 plants per population. The top portions of unopened flower buds, containing anthers and stigma, were sliced off with a razor. Heads were then bagged to prevent loss of achenes. Plants with nearly complete seed set after this treatment were considered to be agamospermous. However, the presence of a few seeds in this set which arose by sexual reproduction cannot be excluded.

Allozyme analysis. Young leaves were ground in extraction buffer according to Kato (1987) with the following buffer: 0.1 M Tris-HCl (pH 8.0), 70 mM mercaptoethanol, 26 mM sodium metabisulphite, 11 mM L-ascorbic acid, 4% soluble PVP. The pH was adjusted after the addition of the ascorbate. Crude homogenates were centrifuged for 10 min at 15 000 rpm. The cleared supernatant was either stored in deep freeze at −70 °C or immediately loaded on the gel. The PAGE was carried out using separating gel (8.16%) with the buffer 1.82 M Tris-HCl, pH 8.9; the spacer gel (4.0%) with the buffer (0.069 M Tris-HCl, pH 6.9); the electrode buffer was 0.02 M Tris, 0.24 M glycine, pH 8.3. The following enzymes were analysed: AAT (Aspartate aminotransferase, EC 2.6.1.1, dimeric), LAP (Leucine aminopeptidase, EC 3.4.11.1, monomeric), PGM (Phosphoglucomutase, EC 5.4.2.2, monomeric), SKD (Shikimate dehydrogenase, EC 1.1.1.25, monomeric). They were visualized with staining procedures generally following Vallejos (1983) with some exceptions – the LAP buffer was replaced by 0.2 M Tris-maleate, pH 6.0. PGM was stained with the agar overlay method. The interpretation of allozyme profiles was based on the analysis of allozyme patterns in populations of diploid sexual *Hieracium alpinum* (Chrtek et Kirschner, unpubl.). No allozyme analysis of the offspring was performed.

Four allozyme systems representing 7 allozyme loci were analyzed electrophoretically, four loci showed interpretable variation. Ten allozyme genotypes were detected in 131 individuals studied.

DNA extraction and RAPD. Fresh or field-collected (and preserved in a NaCl/CTAB solution) leaves were used for DNA extraction as described by Štorchová et al. (2000). Briefly, about 200 mg of leaf tissue was ground in the extraction buffer (0.34 M sorbitol, 0.1 M Tris-HCl pH 7.6, 5 mM EDTA, 0.2% v/v 2-mercaptoethanol) and centrifuged. The pellet was suspended in the extraction buffer, the same volume of the lysis buffer (0.2 M Tris-HCl pH 7.6, 2 M NaCl, 0.05 M EDTA, 2% CTAB) was added, then chloroform extraction, isopropanol precipitation and washing with 80% ethanol were performed.

The PCR reaction was performed using a Gene E Thermocycler (Techne). For each sample at least two reactions per primer with different template concentrations were done. Three or six ng of purified genomic DNA per reaction was used in a final volume of 25 µl under the following conditions: 200 µM of each dNTP (MBI Fermentas), 1.5 mM $MgCl_2$, 0.2 µM primer, magnesium-free reaction buffer and 1 U *Taq* DNA polymerase (Promega) per reaction. After initial heating for 5 min at 94 °C, samples were PCR amplified using 40 cycles (94 °C 20 s, 39 °C 20 s, 72 °C 1 min) followed by a final extension of the PCR products for 4 min at 72 °C. The products of amplification were analysed by electrophoresis in 1.3% agarose gels with 0.5 × TBE running buffer (0.045 M Tris-borate, 1 mM EDTA pH 8.0), visualised by ethidium bromide staining and photographed under UV light with Pentacon camera. Band scoring was performed manually. Only those RAPD markers that reproduced consistently across all PCR reactions were included into the binary matrix.

Twenty decamer oligonucleotides from the kit A (Advanced Biotechnologies, GB) were tested for amplification and polymorphism. Sixteen primers produced reproducible patterns, 4 of them (ABA-11, ABA-18, ABA-19, ABA-20) provided polymorphic bands (Table 2).

186 individuals were studied by RAPD, 91 individual plants were analysed using both RAPD and allozymes.

Data analysis. The allozyme genotype of each individual plant was determined on the basis of its pattern after electrophoresis. The data set of allozyme genotypes (Table 3) was used to calculate

Table 2. Primers selected for RAPD analysis of 186 individuals of *Hieracium* sect. *Alpina*

Primers ABA	Sequence 5′ – 3′	Molecular weights of RAPD fragments (bp)	Number of bands generated	Number of polymorphic bands	Number of distinct RAPD patterns
11	CAATCGCCGT	1900 – 680	9	8	11
18	AGGTGACCGT	770 – 250	4	3	4
19	CAAACGTCGG	2150 – 520	9	5	6
20	GTTGCGATCC	1750 – 590	6	3	4
Total			**28**	**19**	

Table 3. Allozyme genotypes and their frequencies found in the examined species. Genotype frequencies are calculated within the given species

Species [number of plants]	2n	*Skd*	*Lap-1*	*Pgm-1*	*Aat-2*	Genotype	Frequency of genotype [number of plants]
H. alpinum [40]	27	aaa	acc	bbc	ccc	I	0.025 [1]
	27	aaa	ccc	bbc	aac	II	0.100 [4]
	27	aaa	ccc	bbc	ccc	III	0.525 [21]
	27	abb	ccc	bcc	aac	IV	0.350 [14]
H. crassipedipilum [17]	27	aab	ccc	bbb	acc	V	0.706 [12]
	27	aaa	acc	bbc	ccc	I	0.294 [5]
H. pinetophilum [21]	27	aaa	acc	bbc	bcc	VII	0.571 [12]
	27	aaa	ccc	bbc	acc	VIII	0.429 [9]
H. krivanense [17]	36	abcc	accc	bbbb	accc	IX	
H. halleri [26]	27	aac	cce	bbc	ccd	X	
H. slovacum [10]	36	aabc	ccce	abbc	accd	XI	

frequency of alleles, the mean number of alleles per locus within each population (A), and the within-population observed heterozygosity (Ho). Since populations of various ploidy levels were studied, the genotype frequencies were used to estimate other genetic diversity values. They were calculated within each population, species, and for the total material and were used for estimation of allozyme diversity at different hierarchical levels as $G(pop/sp/gen) = 1 - \Sigma g_i^2$ (using the formula as in Stace et al. 1997), where g_i is the frequency of the ith genotype within a population, a species or in the total plant sample. Gsp′ was calculated as a mean of Gpop values within a species. The mean of Gsp (among all species) was determined as Ggen′. The partitioning of allozyme variance among populations within a species or among species within the whole plant sample was estimated as $Gst = 1-(Gsp'/Gsp)$ and $Gst(sp) = 1-(Ggen'/Ggen)$, respectively. Finally, we used the matrix of allele frequencies within populations and species to compute pairwise Euclidean distances among populations.

RAPD markers are dominant, therefore a direct estimation of observed heterozygosity is not possible. Calculation of allele frequencies always depends on assumptions of Hardy-Weinberg equilibrium within populations (Lynch and Milligan 1994), which are not relevant for apomictic and polyploid plants. Thus, we restricted our analysis of RAPD data to analysis of phenotypes, represented by presence or absence of a RAPD marker in each individual. A binary matrix of RAPD marker phenotypes (Appendix 2) was used to calculate frequencies of markers within populations and species. Based on these frequencies, we calculated Shannon's index of diversity within populations and species and partitioning of molecular variance among populations and species, as described in Bussell (1999) and Bartish et al. (2000). The same data sets of RAPD phenotype frequencies were used also to compute the matrices of pairwise Euclidean distances among populations.

In our analysis of molecular data sets we refrained from application of phylogenetic algorithms, such as parsimony analysis. As hybridizations in the evolution of *Hieracium* species cannot be excluded, we must take reticulation events into consideration. Reticulate evolution is not treated properly by cladistic methods. Therefore, we used ordination procedures to obtain a representation of taxonomic relationships within a group of closely related plants subjected to polyploidization and apomixis. We performed the PCO analyses on both matrixes of pairwise Euclidean distances among populations (based on RAPD phenotypes and allozyme genotypes) by application of NTSYS software (NTSYS-pc, version 1.8, Rohlf 1997), programs DCENTER and EIGENVECTOR. The results of this analysis were presented as three-dimensional plots of the first three principle components.

The product-moment correlation and the Mantel test statistics were computed with NTSYS (MXCOMP program) to estimate the relationship between the allozyme and RAPD distance matrices. For this comparison (of distances among populations), only the distances among populations, analysed by both methods, were considered. A similar approach was applied to analyse possible correlations between genetic (based on allozyme data set) or phenetic (RAPD data set) distances (on the one hand), and geographic distances (on the other hand), for all pairwise comparisons among populations within *H. alpinum*, *H. crassipedipilum* (only RAPD data), and the whole plant sample.

Results

Cytology. We report here for the first time the chromosome number in *H. krivanense* ($2n = 36$, populations Kriv 1, Kriv 2, Kriv 3, see Appendix 1, 17 plants in total). Previously reported chromosome counts (Chrtek and Marhold 1998, Skalińska et al. 1959, Májovský 1970) were confirmed for *H. alpinum* ($2n = 27$), *H. halleri* ($2n = 27$), *H. pinetophilum* ($2n = 27$) and *H. slovacum* ($2n = 36$). The chromosome number in *H. crassipedipilum* ($2n = 27$, all plants studied) differed from the previously reported $2n = 36$ (Chrtek and Marhold 1998).

Pollen production and mode of reproduction. Pollen production was observed in *H. krivanense* and *H. slovacum* (in all plants examined), whereas the remaining species lacked pollen. The percentage of fertile (dark brown to black) achenes in the primary head

Table 4. Mean within species estimates of genetic diversity within populations of *Hieracium* computed from allozyme genotypes (Ho, A, and Gsp′) and RAPD phenotypes (Shannon's index) and partitioning of total molecular variance within species of *Hieracium*. Gst is the component of total variance, attributed to variation among populations. Values in parentheses are standard deviations for correspondent mean estimates, where variability among values was observed (standard deviations for Gst in allozymes cannot be determined since only one estimate is available for each species)

Species	Allozymes						RAPD		Alloz.	RAPD	
	Ho		**A**		**Gsp′**		**Shannon's index**		**Gst**	**Gst**	
H. alpinum	0.47	(0.25)	1.47	(0.25)	0.11	(0.21)	0.05	(0.04)	0.81	0.77	(0.28)
H. halleri	1.00	n.a.[a]	2.00	n.a.	0.00	n.a.	0.02	(0.03)	0.00	0.46[c]	
H. crassipedipilum	0.50	n.a.	1.50	n.a.	0.00	n.a.	0.00	n.a.	1.00	1.00	n.a.
H. pinetophilum	0.70	(0.11)	1.75	(0.18)	0.09	(0.21)	0.00	n.a.	0.79	1.00	n.a.
H. krivanense	0.75	n.a.	2.00	n.a.	0.00	n.a.	0.00	n.a.	0.00	1.00	n.a.
H. slovacum	1.00	n.a.	2.75	n.a.	0.00	n.a.	0.00	n.a.	ND[b]	ND	

[a] n.a. – standard deviations are not available due to uniformity in observed values;
[b] ND (not determined) – only one population available;
[c] estimate is based on one polymorphic marker only

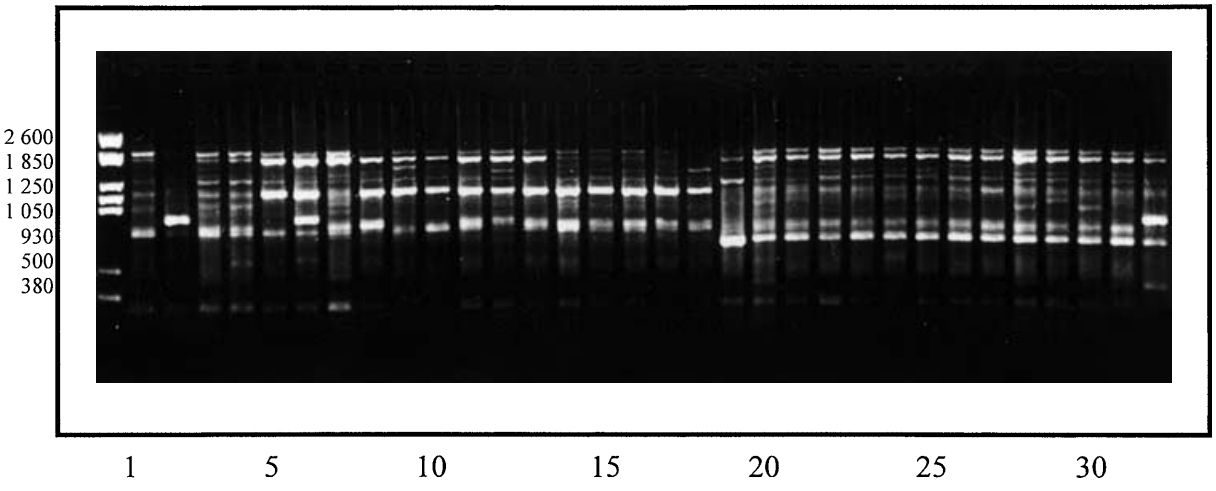

Fig. 2. Comparison of the RAPD patterns of the plants representing various populations of *Hieracium* sect. Alpina, obtained with the primer ABA-11. **1–7** *H. alpinum* s.str. (**1** Alp-1, **2** Alp-1 – other individual, **3** Alp-2, **4** Alp-3, **5** Alp-4. **6** Alp-4 – other individual, **7** Alp-5), **8–12** *H. crassipedipilum* (**8** Cras-1, **9** Cras-2, **10** Cras-3, **11** Cras-4, **12** Cras-5), **13–18** *H. pinetophilum* (**13** Pin-1, **14** Pin-2, **15** Pin-3, **16** Pin-4, **17** Pin-5, **18** Pin-6), **19** *H. krivanense* Kriv-1, **20** *H. slovacum* Slov-1, **21–32** *H. halleri* (**21** Hal-1, **22** Hal-1 – other individual, **23** Hal-2, **24** Hal-3, **25** Hal-4, **26** Hal-4, **27** Hal-6, **28** Hal-7, **29** Hal-8, **30** Hal-9, **31** Hal-5, **32** Hal-5 – other individual). Molecular weight standard (pBR322 DNA – *Alw*44I/*Mva*I digest) is loaded on the left side of the gel

did not vary substantially among species: *H. pinetophilum* 82–91%, *H. crassipedipilum* 84–96%, *H. slovacum* 90–97%, *H. krivanense* 78–92%. The proportion of fertile achenes in *H. alpinum* and *H. halleri* were the same as in these species (Chrtek 1997, similarly to present results agamospermy was proven).

Polymorphism and genetic diversity at different hierarchical levels. Analysis of allozyme genotypes revealed high heterozygosity and a

relatively high number of alleles per locus (Table 4). At the species level, overall heterozygosity and the mean number of alleles per locus within populations was lowest in *H. alpinum* (Ho = 0.47 and A = 1.47) and highest in *H. slovacum* (Ho = 1 and A = 2.75).

Sixteen primers employed in this study provided a total of 62 repeatable RAPD fragments within 40 populations (186 individuals). Four primers (Table 2) produced 19 polymorphic markers (31% of all scorable markers). A total of 16 different RAPD phenotypes were distinguished (Fig. 2).

Both allozyme and RAPD methods revealed low within-population diversity in all studied species. In *H. alpinum*, within-population variability was found in several populations (mean Gsp′ = 0.11 for allozyme genotypes and mean Shannon's index = 0.05 for RAPD phenotypes, Table 4). Some diversity was found also within several populations of *H. pinetophilum* by allozyme analysis (Gsp′ = 0.09) and in *H. halleri* by RAPD analysis (Shannon's index = 0.02). No within-population diversity was found in the three remaining species (Fig. 2).

Among population differentiation was found in *H. alpinum*, *H. crassipedipilum*, and *H. pinetophilum* in both allozyme and RAPD analyses. RAPD's also showed differentiation among populations in *H. krivanense* and *H. halleri*, where allozyme variation was absent. Partitioning of molecular variance into within- and among-population components showed that most of variance was allocated among populations (Table 4). When the total variance was partitioned into within- and among-species components, most of it was attributed to variation among species (72% and 78% for estimations based on allozyme and RAPD data sets, respectively).

We found a moderate but highly significant (Pearson coefficient of correlation, $r = -0.51$; $P < 0.001$ in t-test) negative correlation between values of observed within species heterozygosity (Ho) and the mean values of genetic diversity within populations for all species (Gsp′ for allozymes in Table 4). Populations which are exclusively apomictic could have arisen from past hybridization events. Their heterozygosity might have become fixed owing to the asexual mode of reproduction.

PCO analysis of genetic differentiation between populations. Three-dimensional representation of PCO analysis of Euclidean distances among populations, computed from within-population allele frequencies of allozymes, revealed several distinct groups of populations (Fig. 3A). One of these groups was heterogeneous and consisted of populations from three species: *H. alpinum* (populations 1–4, 6, 8), *H. crassipedipilum* (population 1), and *H. pinetophilum* (all populations). All other distinct groups represented populations of the same species: *H. alpinum* (populations 5, 7), *H. crassipedipilum* (populations 2, 9, 11), *H. halleri* (all populations), *H. krivanense* (all populations) and *H. slovacum*.

The results of the PCO analysis of Euclidean distances among a larger sample of populations of the same species, based on the frequencies of RAPD phenotypes (Fig. 3B), were generally congruent with the PCO analysis of the data set of allozyme genotypes. Two populations of *H. alpinum* (populations 5, 7), *H. halleri* (identical to *H. slovacum*) and *H. krivanense* were again distinct entities, whereas most populations of *H. alpinum* (populations 1–4), together with all populations of *H. crassipedipilum*, represented a heterogeneous group of populations. In contrast to the results of PCO analysis based on allozymes, most populations of *H. pinetophilum* (all except populations 1 and 9) were clearly distant to this heterogeneous group and appeared to be more closely related to *H. krivanense*. It is noteworthy, that those two populations of *H. pinetophilum*, 1 and 9, and population 10 and 4 of *H. crassipedipilum*, share an identical RAPD phenotype.

Euclidean distance matrices obtained from pairwise comparisons between the populations, analyzed by RAPD and allozyme data (24 populations), were significantly correlated in Mantel test ($r = 0.610$, $P < 0.001$).

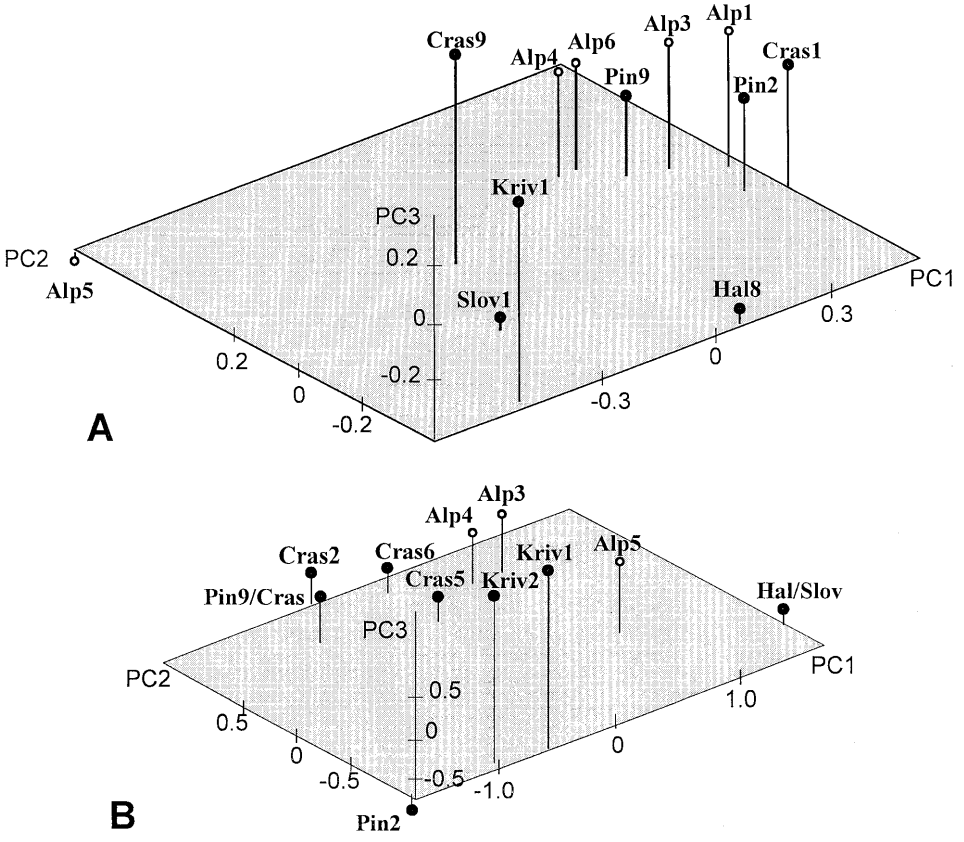

Fig. 3. Three-dimensional representation of principal co-ordinate analysis of phenetic relationships between representative populations of *Hieracium* sect. *Alpina*. Percentage of total variance explained variability was for **A** the allozyme based dataset: PC1 38.3%, PC2 26.8% and PC3 19.8%. **B** the phenetic RAPD-based dataset: PC1 45.2%, PC2 30.1%, PC3 9.0%

Geographic structure of genetic differentiation among populations. A Mantel test of correlations between pairwise Euclidean distances and geographical distances among all studied populations (estimated from allozyme and RAPD data) was performed. No significant correlation for both allozyme ($r = 0.017$, one-tailed t-test: $P = 0.619$), and RAPD ($r = 0.029$, one tailed t-test: $P = 0.179$) data was revealed. When the same analysis was performed for populations of *H. alpinum* separately, a marginal significance for correlation between geographic and Euclidean distances was found for both allozyme ($r = 0.639$, $P = 0.041$), and RAPD markers ($r = 0.331$, $P = 0.074$). Populations of *H. alpinum* of the Západné Tatry Mts. (1, 2, 3) differ considerably from populations of the Belanské Tatry Mts. (7) in both morphological and molecular characters. Populations Alp 4 found in Vysoké Tatry Mts., in the middle of the Tatry Mts., has the highest level of genetic diversity among studied populations. It shows similarities with both the populations from western as well as from eastern parts of the Tatry Mts.. In contrast to *H. alpinum*, no significant correlation between geographic and Euclidean distances based on RAPDs ($r = 0.070$, $P = 0.413$) was revealed among populations of *H. crassipedipilum*, which were analysed separately.

Discussion

Genetic variation within *H. alpinum*. Little is known about the details of the speciation

process in *Hieracium*. This genus involves many apomictic species differing in minute but stable morphological characters, enabling taxonomical determination. It is unclear whether the large number of morphologically distinct taxa arose solely through somatic mutations or may also be influenced by gene flow from extant sexual ancestors. It has been documented that some *Hieracium* species, consisting of apomicts, include diploid sexual populations (Chrtek 1997). These sexual plants may have hybridized with plants producing seeds both apomictically and sexually. *H. alpinum* is a species comprised of both apomictic and sexual populations. While in the Eastern Carpathians only sexual diploids have been found so far, commonly occurring agamospermous triploids are known from the remaining part of their distribution, including the Tatry Mts.. Tetraploids of *H. alpinum* (Szeląg and Jankun 1997) may represent an important link between the East Carpathian diploid cytotype and the widespread triploids.

Despite the apomictic mode of reproduction, considerable genetic variation was revealed in *H. alpinum* by allozyme as well as by RAPD analysis in a very limited geographical area of the Tatry Mountains. This species shows the highest degree of genetic diversity among the taxa studied. The observed genetic differences are generally congruent with morphological observations. Although Zahn (1922–1938) reported 7 subspecies of *H. alpinum* (of *H. alpinum* "Gruppe" *alpinum*) in the Tatry Mts., we observed a mosaic-like distribution of morphological characters representing within-population variation.

A significant correlation between genetic distances among populations of *H. alpinum*, based on allozyme or RAPD data, and geographical distances among corresponding populations was found in the Tatry Mts.. Such results are typical for outcrossing species as has been described recently (Nybom and Bartish 2000) and may represent isolation by distance.

Shi et al. (1996) and Stace et al. (1997) reported genetic variation in *H. alpinum* from Great Britain and Switzerland, but to a much lower degree than we found in the small area of the Tatry Mts.. Shi et al. (1996) discovered five allozyme genotypes in 56 plants and within-population variation at three loci. They found no clear differences between allozyme profiles of the Scottish and Swiss populations. Differences between Scottish and Swiss plants were demonstrated by RAPD, but no variation was detected within countries. RAPD data correlated well with morphological studies which showed differences between plants from Scotland and Switzerland.

Putative glacial refugium and postglacial migration of *H. alpinum*. Along with others, we recognize a gradient of genetic diversity of *H. alpinum* in Europe ranging from east (variable diploid populations in the Eastern Carpathians) to west (uniform populations of Great Britain) (Shi et al. 1996, Stace et al. 1997, our unpublished data). This spatial pattern of genetic variation can be related to possible routes of colonization in postglacial times, supposing that there was a glacial refugium in the Eastern Carpathians. The reduction of genetic diversity with increasing geographic distance from the refugium could be explained by repeated population bottlenecks due to rare long-distance seed dispersal with subsequent founder effects (Comes and Kadereit 1998). The total range of *H. alpinum* extends from Greenland, through Iceland, the British and Scandinavian mountains, to North-West Russia and includes the Alps, the Sudeten Mts., the Carpathians, and isolated exclaves in the Vosges, Harz and at the Vranica plateau (Bosnia and Hercegovina), (Bräutigam 1992). The only region where diploid *H. alpinum* occurs, is the Eastern Carpathians. Only triploid apomictic plants have been found in the remaining parts of the arcto-alpine distribution area. As diploid populations are assumed to be ancestors of triploid and tetraploid *H. alpinum*, their occurrence in Eastern Carpathians represents additional

support for the location of a glacial refugium of this species in this mountain range.

The alpine populations of the Eastern and Western Carpathians have been disjunct for several thousands of years, but tundra communities could migrate during some periods of Ice Age (the Tatry Mts. and the highest parts of the Eastern Carpathians were covered by local mountain glaciers). Thus, immigration of sexual diploids (or tetraploids) and some level of outbreeding cannot be excluded.

The location of glacial refugia in Europe and routes of postglacial migration of many plant species have been described (see Comes and Kadereit 1998 for review). Unfortunately, mountain plants of the Carpathians have rarely been included in biogeographic studies using molecular markers (Hungerer and Kadereit 1998.)

Genetic variation within *H. halleri* and species of the *H. fritzei*-group. Low levels of genetic variation were found in *H. halleri* and species of the *H. fritzei*-group. These species are generally each confined to one mountain system and thus they have smaller distribution areas than the arcto-alpine *H. alpinum* (Fig. 1).

H. halleri occurs in the Alps and the Western Carpathians. The genetic diversity of the whole set of plants of *H. halleri* from the Tatry Mts. was near zero (two RAPD phenotypes and one allozyme genotype in 9 populations). The results are consistent with morphology – there is very low variation in taxonomically important characters (e.g. type of indumentum). In contrast, populations of *H. halleri* from the Alps are morphologically more variable (Chrtek jun., unpublished). It would be very interesting to compare molecular markers of *H. halleri* populations from the both mountain ranges. Similar to *H. halleri*, samples of *H. krivanense* show very low genetic variation (two RAPD phenotypes and one allozyme genotype). The uniformity of *H. slovacum* (only one genotype) agrees with results obtained for other geographically restricted endemics with an agamospermous mode of reproduction.

Molecular variation in *H. crassipedipilum* is solely due to variation among populations (Table 4). In contrast to *H. alpinum*, no significant correlation between geographic and genetic distances among populations was revealed in *H. crassipedipilum*. Unlike other agamic species studied here, it represents a morphologically variable taxon, presumably consisting of genetically different lineages. These patterns are consistent with the populations of *H. crassipedipilum* arising independently and, after the initial hybridization events, the plants propagating exclusively by apomixis, conserving fixed heterozygosity.

In *H. pinetophilum*, both RAPD and allozyme studies revealed small differences between populations from the western and eastern part of the Tatry Mts.. Plants of both phenotypes were found in population 9, collected near the tentative border between the Západné Tatry Mts. and the Vysoké Tatry Mts. (Mlynická dolina valley). Morphologically "pure" *H. pinetophilum* is (with some exceptions) confined to the eastern parts of the Tatry Mts.. In the western part, it is sometimes difficult to draw a line between *H. pinetophilum* and *H. crassipedipilum*.

Similar to our findings in *H. halleri*, *H. krivanense* and *H. slovacum*, only one multi-locus allozyme genotype was found by Stace et al. (1997) in each of 25 agamospermous taxa of section *Alpina* in Great Britain (however, 12 of 36 taxa were studied in one population only). The uniclonal structure of British taxa was in agreement with the low level of morphological variation that they found. Stace et al. (1997) concluded that molecular variation is not necessarily greater in widely distributed taxa, because no variation was observed in *H. holosericeum* Backh., which is widespread in Scottish, Welsh and English mountains.

Relationships among *Hieracium* species examined. Our study shows that the species of the *H. fritzei*-group in the Tatry Mts. have at least two progenitors. Unlike the other taxa,

endemic tetraploid *H. slovacum* shares all the unique allozyme alleles of triploid *H. halleri* (Table 3) and all RAPD patterns of the two species are identical (Fig. 3B). This is consistent with *H. slovacum* and *H. halleri* sharing one parent. The contribution of another progenitor can be recognized in the allozyme pattern of *H. slovacum* (unique allele of *Pgm*-1). We succeeded to collect just one population of *H. slovacum* for molecular studies, therefore more populations should be found and analyzed before final conclusion concerning this species can be drawn. In contrast, *H. halleri* probably did not participate in the origin of *H. pinetophilum* and *H. crassipedipilum*. PCO based on RAPD and allozyme data placed populations of *H. crassipedipilum*, *H. pinetophilum* and *H. alpinum* into one group clearly separated from *H. halleri* and *H. krivanense* (Fig. 3A, 3B). Therefore it is reasonable to consider that *H. alpinum* might be the ancestral taxon of *H. crassipedipilum* and *H. pinetophilum*. Based upon our data, Carpathian species of the *H. fritzei*-group cannot be derived from a single ancestor. Besides *H. alpinum*, *H. halleri* may also have participated in evolution of *H. fritzei*-group.

Apomictic species of *Hieracium* and other genera. Tyler (2000) attempted to use apomictic species (microspecies, agamospecies) for phylogeographic analysis by studying the distribution of 325 species belonging to two *Hieracium* sections (*H.* sect. *Hieracium* and *H.* sect. *Vulgata*) in the Nordic countries. These studies concluded that geographic patterns in species composition could reflect historical migration routes. We found medium levels of between- and even within-population genetic variations, estimated from RAPD data in apomictic species of *H.* sect. *Alpina*. Four RAPD phenotypes were recognized in *H. crassipedipilum*, two RAPD phenotypes were found in each *H. halleri*, *H. pinetophilum*, *H. krivanense* (Appendix 2). It cannot be excluded that even morphologically uniform entities recognized at the microspecies level may have been genetically heterogenous.

Similar to *Hieracium* s.str., the genus *Taraxacum* lacks sexual reproduction over large parts of its area and reproduces only by meiotic diplospory (diplospory of Taraxacum type). Investigations of enzyme polymorphism revealed some agamospecies to be nearly invariable, whereas others, such as *T. vindobonense* Soest, show high levels of enzymatic diversity (Battjes et al. 1992, Hughes and Richards 1988, Kirschner and Štěpánek 1994, Menken et al. 1995). Wittzell (1999) detected no variation within each of 12 agamic species belonging to various *Taraxacum* sections (sequencing of the *trn*L-*trn*F intergenic region in cpDNA). Considerable genetic variation was revealed within populations of apomictic dandelions using DNA markers (King 1993, Van der Hulst et al. 2000). Hybridization between sexual diploids and apomictic triploid plants producing diploid polled grains was proposed to explain this variations.

Many studies have been performed in species comprising both sexual and agamospermous populations (or in closely related sexual and agamospermous species). Considerable genetic variation was found e.g. in facultatively agamospermous *Amelanchier laevis* Wieg. (Campbell et al. 1999). Few highly heterozygous genotypes with some alleles not detected in diploid sexual populations were found in agamospermous populations of *Erigeron compositus* Porsh (Noyes and Soltis 1996). These studies suggest that broad generalization of genetic variation in agamospermous taxa is not possible. Instead, differences are taxon specific and could be strongly influenced by the frequency of sexual reproduction, genetic divergence of progenitor taxa and the extent of mutational changes in somatic tissues.

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Appendix 1. Populations of the *Hieracium alpinum* and *H. fritzei* groups studied. Abbreviations of species are as follows: Alp – *H. alpinum*, Cras – *H. crassipedipilum*, Pin – *H. pinetophilum*, Kriv – *H. krivanense*, Hal – *H. halleri*, Slov – *H. slovacum*. Abbreviations of geographical areas: ZT – Západné Tatry Mts., VT – Vysoké Tatry Mts., BT – Belanské Tatry Mts. (cf. Fig. 1)

	Localities
Alp 1	ZT: Mt. Baníkov, slopes abote the Baníkovské sedlo saddle, ca 9.5 km SE of Zuberec, 2080 m alt., 19°42′35″ E 49°12′00″ N
Alp 2	ZT: Mt. Hrubá kopa, 10.2 km SE of Zuberec, 2160 m alt., 19°43′30″ E, 49°12′03″ N
Alp 3	ZT: Roháčske plesá mountain lakes, around the uppermost lake, ca 10 km SE of Zuberec, 1720 m alt., 19°44′10″ E, 49°12′25″ N
Alp 4	VT: Malá Studená dolina valley Päť Spišských plies mountain lakes, 6 km NNW of Starý Smokovec, 2040 m alt., 20°11′45″ E, 49°11′35″ N
Alp 5	VT: Mlynická dolina valley, above the waterfall Skok, 4.2 km NNW of Štrbské Pleso, 1770 m alt., 20°02′50″ E, 49°09′15″ N
Alp 6	ZT: Račkove plesá mountain lakes, slopes near the lakes, 11 km N of Pribylina, 1725 m alt., 19°48′38″ E, 49°12′00″ N
Alp 7	BT: Kopské sedlo saddle, 7 km SE of Tatranská Javorina, 1760 m alt., 20°13′15″ E, 49°13′50″ N
Alp 8	ZT: Mt. Hrubý vrch, W slopes, 11.5 km N of Pribylina, 1980 m alt., 19°47′38″ E, 49°11′58″ N
Cras 1	ZT: Roháčske Plesá mountain lakes, around the uppermost lake, ca 10 km SE of Zuberec, 1720 m alt., 19°44′10″ E, 49°12′25″ N
Cras 2	ZT: Mt. Roh (mountain ridge between Mt. Lúčna and Mt. Osobitá), 20.5 km ESE of Zuberec, 1560 m alt., 19°45′32″ E, 49°14′25″ N
Cras 3	BT: Predné Med'odoly valley, 6 km W of Tatranská Kotlina, 1540 m alt., 20°14′20″ E, 49°13′30″ N
Cras 4	VT: Mlynická dolina valley, 3.5 km NNW of Štrbské Pleso, 1600 m alt., 20°02′55″ E, 49°09′00″ N
Cras 5	VT: Mlynická dolina valley, near the waterfall "Skok", 4 km NNW of Štrbské Pleso, 1680 m alt., 20°02′55″ E, 49°09′10″ N
Cras 6	VT: Mengusovská dolina valley, 5.5 km N of Štrbské Pleso, 1700 m alt., 20°04′00″ E, 49°10′05″ N
Cras 7	VT: Popradské pleso mountain lake, grasslands on the south bank, 4 km NNE of Štrbské Pleso, 1495 m alt., 20°05′00″ E, 49°09′10″ N
Cras 8	BT: Monkova dolina valley, 4.8 km SW of Ždiar (church), 1760 m alt., 20°13′00″ E, 49°14′35″ N
Cras 9	VT: Mt. Kriváň, SW slopes, 5 km E of Podbanské, 1360 m alt., 19°59′00″ E, 49°09′20″ N
Cras 10	VT: Velická dolina valley, 5.5 km NW of Starý Smokovec, 1800 m alt., 20°09′25″ E, 49°09′44″ N
Cras 11	VT: Furkotská dolina valley, 4.7 km NW of Štrbské Pleso, 1900 m alt., 20°01′50″ E, 49°09′15″ N
Pin 1	ZT: Smutná dolina valley, 11.3 km SE of Zuberec, 1440 m alt., 19°45′10″ E, 49°12′45″ N
Pin 2	VT: Vel'ké Biele Pleso mountain lake, 7.2 km NW of Tatranská Lomnica,1600 m alt., 20°14′10″ E, 49°13′25″ N
Pin 3	VT: Malá Studená dolina, 5.2 km NNW of Starý Smokovec, 1840 m alt., 20°12′30″ E, 49°11′45″ N
Pin 4	VT: Vel'ká Studená dolina, 5.2 km NW of Starý Smokovec, 1730 m alt., 20°10′45″ E, 49°10′30″ N
Pin 5	VT: Mlynická dolina valley, 3.6 km NNW of Štrbské Pleso, 1650 m alt., 20°02′55″ E, 49°08′53″ N

Appendix 1 (continued)

	Localities
Pin 6	ZT: Račkove plesá mountain lakes, S slopes of Mt. Končistá above the lakes, 11.7 km N of Pribylina, 1780 m, 19°48′42″ E, 49°12′05″ N
Pin 7	VT: Mengusovská dolina valley, 5.5 km N of Štrbské Pleso, 1740 m alt., 20°04′00″ E, 49°10′05″ N
Pin 8	VT: Mengusovská dolina valley, 5.2 km N of Štrbské Pleso, 1630 m alt., 20°04′10″ E, 49°09′55″ N
Pin 9	VT: Mlynická dolina valley, near the waterfall ''Skok'', 4 km NNW of Štrbské Pleso, 1690 m alt., 20°02′55″ E, 49°09′03″ N
Pin 10	BT: Monkova dolina valley, 4.8 km SW of Ždiar (church), 1760 m alt., 20°13′00″ E, 49°14′35″ N
Pin 11	VT: Velické pleso mountain lake, grasslands on western bank, 5.3 km NWW of Starý Smokovec, 1670 m alt., 20°09′25″ E, 49°09′30″ N
Kriv 1	ZT: Mt. Roh (mountain ridge between Mt. Lúčna and Mt. Osobitá), 20.5 km ESE of Zuberec, 1560 m alt., 19°45′32″ E, 49°14′25″ N
Kriv 2	VT: Furkotská dolina valley, 3.5 km NW of Štrbské Pleso, 1700 m alt., 20°02′18″ E, 49°08′35″ N
Kriv 3	VT: Mt. Kriváň, SW slopes, 5 km E of Podbanské, 1360 m alt., 19°59′00″ E, 49°09′20″ N
Hal 1	ZT: Mt. Kansse, 9 km E of Zuberec, 1460 m alt., 19°44′20″ E, 49°15′25″ N
Hal 2	VT: Kôprová dolina valley, Nižné Temnosmrečianske pleso mountain lake, 8.5 km NNW of Štrbské Pleso, 1690 m alt., 20°12′10″ E, 49°11′35″ N
Hal 3	VT: Skalnaté pleso mountain lake, 4.5 km NW Tatranská Lomnica, 1720 m alt., 20°14′10″ E, 49°11′30″ N
Hal 4	ZT: Roháčske plesá mountain lakes, 10.5 km SE of Zuberec, 1650 m alt., 19°44′20″ E, 49°12′27″ N
Hal 5	VT: Mlynická dolina valley, 3.6 km NNW of Štrbské Pleso, 1650 m alt., 20°02′55″ E, 49°08′53″ N
Hal 6	ZT: Mt. Lúčna, SW slopes, 11 km EES of Zuberec, 1560 m alt., 19°45′55″ E, 49°14′10″ N
Hal 7	ZT: Mt. Osobitá, S slopes, 7.8 km E of Zuberec, 1580 m alt., 19°43′30″ E, 49°15′45″ N
Hal 8	VT: Vel'ká Studená dolina valley, 6 km NW of Starý Smokovec, 1980 m alt., 20°10′05″ E, 49°10′45″ N
Hal 9	VT: Mlynická dolina valley, 3.2 km NNW of Štrbské Pleso, 1600 m alt., 20°03′03″ E, 49°08′40″ N
Slov 1	BT: Kopské sedlo saddle, 5.5 km SW of Ždiar (church), 1735 m alt., 20°13′05″ E, 49°14′00″ N

Appendix 2. RAPD data matrix

Alp 1A	Alp 1B	Alp 2A	Alp 2B	Alp 3A	Alp 3B	Alp 4A	Alp 4B	Alp 4C	Alp 5	Alp 7	Cras 1	Cras 2	Cras 3	Cras 4	Cras 5	Cras 6	Cras 7	Cras 8	Cras 9	Cras 10	Cras 11	Pin 1	Pin 2	Pin 3	Pin 4	Pin 5	Pin 6	Pin 7	Pin 8	Pin 9	Pin 10	Pin 11	Kriv 1	Kriv 3	Kriv 5	Hal 1A	Hal 1B	Hal 2A	Hal 2B	Hal 3	Hal 4	Hal 5A	Hal 5B	Hal 6	Hal 7	Hal 8	Hal 9	Slov 1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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