Genetic variation in agamospermous populations of *Hieracium echioides* in southern Slovakia and northern Hungary (Danube Basin)

Genetická variabilita agamospermických populací *Hieracium echioides* na jižním Slovensku a v severním Maďarsku (Podunají)

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Six populations of *Hieracium echioides* subsp. *echioides* var. *tauscheri* from the Danube Basin between Bratislava and Budapest (locations: Balinka, Čenkov, Devín, Dorog, Győr, Pilis) were analysed using allozyme and karyological analysis. Five allozyme systems (EST, LAP, 6PGDH, PGM, and SKDH) were used to analyse the genetic structure of the examined populations. Analyses revealed low genetic variation both within- and among populations. Four multilocus allozyme phenotypes were detected; three populations (Čenkov, Devín and Győr) possessed phenotype I exclusively, while phenotype II was found only in the Balinka and Dorog populations. Two different phenotypes were found in the population of Pilis (phenotypes III and IV). However, due to the complex banding patterns generated for EST, allelic interpretation was not possible, and the Balinka and Dorog populations appeared to possess different phenotypes. All populations proved to be tetraploid (2n = 36) and agamospermous. The geographic distribution pattern of the analysed populations (one allozyme phenotype at several isolated localities) may reflect a more common occurrence of the taxon in the past. Landscape changes, caused by changes in human management of the country, may have resulted in a loss of suitable localities, mainly open sandy habitats. These changes may have caused the reduction and fragmentation of *H.* subsp. *tauscheri* habitat.

K e y w o r d s : *Asteraceae*, allozyme analysis, *Compositae*, karyology, agamospermy, *Hieracium echioides*, Danube Basin

Introduction

The genus *Hieracium* L. is taxonomicaly one of the most complicated genera of vascular plants. There are several opinions on the infrageneric classification and the number of species varies from ca 500 to ca 10,000 according to the species concept adopted. In Europe, the genus is divided into two subgenera: subgen. *Hieracium* and subgen. *Pilosella* (Hill) S. F. Gray. *Hieracium* species (in the broad sense) are divided into two groups: (1) so-called basic species (“Hauptarten”, “species principales”), and (2) intermediate species (“Nebenarten”, “Zwischenarten”, “species intermediae”) (e.g. Nägeli & Peter 1885, Zahn 1921–1923). The latter are supposed to result from hybridization between two or more basic species.

Excessive morphological variation in *Hieracium* subgen. *Pilosella* is caused by a combination of common hybridization, agamospermy (aposporous type), and polyploidy (x = 9, diploids to octoploids have been discovered in wild populations) (Krahulcová et al. 2000).

Two or more ploidy levels are often reported to occur within a species (Rotreklová 2004). The newly established hybridogeneous taxa can form large clonal stands through a combination of agamospermy and vegetative spreading (Krahulcová et al. 2000, Bicknell & Koltunow 2004). Apomixis occurs by apospory coupled with autonomous embryo and endosperm formation (Rosenberg 1906, 1907, Asker & Jerling 1992). It is mostly facultative, with both sexual and apomictic processes present within the plant (REFS). Various reproductive pathways (apospory, haploid parthenogenesis, cross-fertilization of both reduced and unreduced female gametes) resulting in progeny with high genetic, morphological and karyological diversity have been reported for several species (Krahulcová & Krahulec 2000, Bicknell et al. 2003, Krahulcová et al. 2004). In addition, the pollen of agamospermous plants is usually viable and may participate in hybridization (Gadella 1982, 1987).

Hieracium echioides Lumn. is a morphologically well defined basic species distributed in Central and E Europe and in the steppe region of Asia. Westwards it reaches E Germany, the Czech Republic and E Austria (Bräutigam 1992). Three ploidy levels, diploids, triploids and tetraploids, have been reported (Rotreklová et al. 2002), and in addition, pentaploids were recently discovered (Rotreklová et al. 2005). All previously studied plants have been shown to be sexual and self-incompatible (Peckert 2001, Rotreklová et al. 2005). Another important feature of note is the lack of both under- and aboveground stolons, making this plant unitary, unlike most other *Hieracium* subgen. *Pilosella*.

Preliminary study of *Hieracium echioides* in S Slovakia (Danube Basin) revealed a population of agamospermous plants (Peckert 2001), matching the morphological description of *H. echioides* subsp. *echioides* var. *tauscheri* Nägeli et Peter. Plants differ from those of the nominal variety in the lower density of simple eglandular hairs on the stems and leaves, less rigid hairs on the whole plant, narrower leaves and smaller flower heads. They start flowering earlier, and the flowering period is shorter. Besides the locality in S Slovakia, plants matching the description of *H. echioides* subsp. *echioides* var. *tauscheri* were found in other localities in the Danube Basin between Bratislava and Budapest (growing mostly on eolic sands) in the herbarium BP (Natural History Museum, Budapest). According to the pilot study, plants of this variety are morphologically rather invariable.

The main objectives of our study were (1) to find out the chromosome number and mode of reproduction in six selected populations of *H.* **tauscheri* in the Danube Basin, and (2) to detect genetic structure of these populations using allozyme analysis, which proved to be an appropriate method for population genetic studies in *Hieracium* subgen. *Pilosella* (Krahulec et al. 2004).

Material and methods

Plants

Plants were collected from six wild populations in S Slovakia and N Hungary in 2002–2003 (Table 1, Fig. 1). Depending on population size, 3–21 plants per population were sampled and cultivated in pots under field conditions in the experimental garden of the Institute of Botany, Průhonice. As the population at the locality Čenkov appears to be under threat of extinction, the leaves were collected directly in the field, stored in a portable ice-box and transported to the Institute of Botany, Průhonice for allozyme analysis. As

Table 1. – Location of samples. All samples were collected by T. Peckert and J. Chrtek jun. See Fig. 1 for geographical location.

Country	Locality	Collection date	Abbreviation
Slovakia	Bratislava: Devín, Sandberg, deserted limestone quarry along the road from Devín to Devínská Nová Ves	14. 6. 1999 11. 6. 2003	Devín
Slovakia	Štúrovo: Čenkov, nature reserve Čenkovská lesostep	11. 7. 2002 11. 6. 2003	Čenkov
Hungary	Székesfehérvár: Balinka, bottom of a deserted limestone quarry near the road from Balinka to Bodajk	9. 7. 2002	Balinka
Hungary	Pilisvörösvár: Pilisszentiván, limestone hill Kalvárie on the NW border of the village	10. 7. 2002	Pilis
Hungary	Dorog: sandy bank ca 200 metres E of flooded sand pit	10. 7. 2002 10. 6. 2003	Dorog
Hungary	Győr: NW border of the town, sands along the road to Komárom	10. 6. 2003	Győr

sampling at the Čenkov locality was non-destructive, all plants from this population were analyzed. Voucher specimens are deposited in herbarium PRC (Charles University, Praha).

Karyology, mode of reproduction, pollen production

Two plants per population were analyzed for chromosome counts. Root tips of mature plants were pretreated with a saturated solution of p-dichlorbenzene for 3 hours at room temperature, fixed in a mixture of acetic acid and ethanol (1:3) overnight and stored in 70% ethanol at 4°C. Root tips were then macerated in a mixture of ethanol and hydrochloric acid (1:1) for 2 min and squashed in a drop of lacto-propionic orceine (Dyer 1963).

The mode of reproduction was tested in two plants per population. The apical part of a young head was cut off with a razor blade in order to prevent pollination (Gadella 1987). Heads were then bagged to prevent loss of ripe achenes. Plants with nearly complete seed set were considered to be agamospermous. Presence/absence of pollen grains was examined in voucher specimens of plants from the analysed populations.

Allozyme analysis

Young leaves of cultivated plants were used for allozyme extractions. Plant material was ground in an extraction buffer following the method of Kato (1987) with some modifications: 0.1M Tris-HCl (pH 8.0), 70mM mercaptoethanol, 26mM sodium metabisulphite, 11mM L-ascorbic acid, 4% soluble PVP, pH adjusted after the addition of the ascorbate. Crude homogenates were centrifuged for 10 min at 15 000 rpm. Clear supernatants were stored at –75°C. PAGE was carried out using a 8.16% gel with the buffer 1.82 M Tris-HCl, pH 8.9; the stacking 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: EST (Esterase, EC 3.1.1.-), LAP (Leucine aminopeptidase, EC 3.4.11.1), PGM (Phosphoglucomutase, EC 5.4.2.2), 6PGDH (6-phosphogluconate dehydrogenase, EC 1.1.1.44), and SKDH (Shikimate dehydrogenase, EC 1.1.1.25).

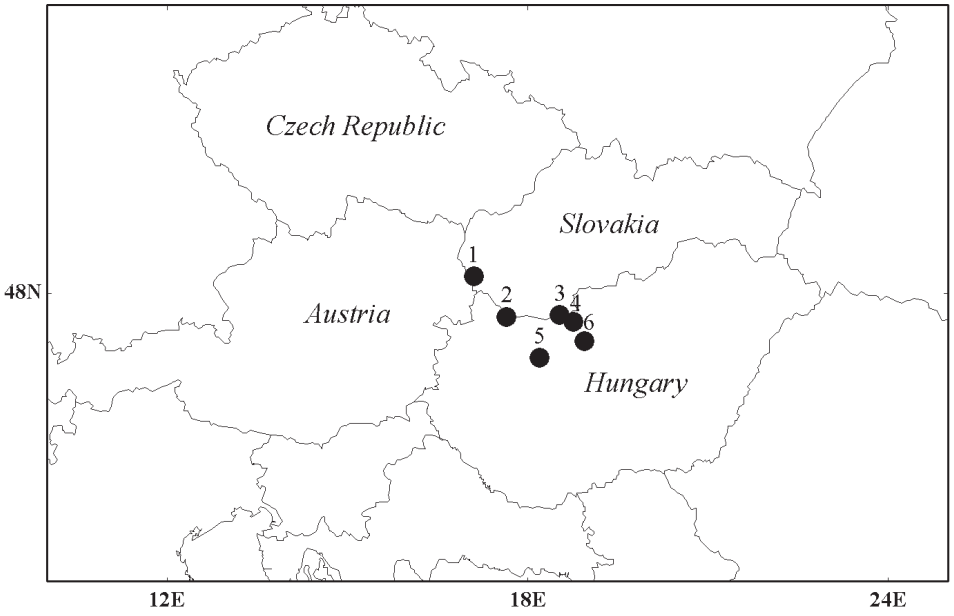

Fig. 1. – Map of Central Europe showing the location of analysed populations: 1 – Devín, 2 – Győr, 3 – Čenkov, 4 – Dorog, 5 – Balinka, 6 – Pilis.

The staining procedures followed Vallejos (1983) to visualize 6-PGDH and SKDH, and Wendel & Weeden (1989) for PGM and EST, with the following modifications: 6-PGDH (0.1 M tris-HCl pH 8.4, 30 mg 6-phosphogluconic acid), SKDH (0.1 M tris-HCl pH 8.4), colorimetric EST (Na-phosphate buffer pH 6.45, 25 mg β-naphthyl phosphate, 50 mg Fast Blue BB), PGM (24 mg $MgCl_2$, 50 mg glucose-1-phosphate, 10 mg NADP). Visualization of LAP was carried out using buffer 0.2 M tris-maleat pH 6. The gel was rinsed with the buffer and then incubated for 10 min in a solution of 30 ml of the buffer, 40 mg L-leucyl-β-naphthylamide.HCl (in 50 % acetone) and 60 mg $MgCl_2$, before 25 mg Fast Black K Salt in 30 ml of the buffer was added. The gel was incubated in the dark until bands appeared.

Banding patterns of LAP, PGM, 6PGDH and SKDH were interpreted allelicaly. The alleles were annotated according to the mobility of corresponding allozymes (a,b…). For EST, only banding patterns were compared. The interpretations of allozyme patterns were based on the analysis of allozyme patterns in populations of diploid sexual *Hieracium echioides* (T. Peckert & J. Chrtek, unpubl.). Mean number of alleles per locus (A), percentage of polymorphic loci (%P), and observed frequency of heterozygotes (H_o) were calculated for each allozyme phenotype/population. Additionally, genotype diversity measures D (genotype diversity within population) and E (genotype evenness, reflecting proportional representations of genotypes within a population sample). For the calculation of D and E see e.g. Noyes & Soltis (1996).

Results

Chromosome counts were made for two plants per population; in the Pilis population counts for both allozyme phenotypes were obtained. All plants were found to be tetraploid (2n = 36). The emasculated heads produced almost full set (average 73.8%) of well developed achenes. Pollen production was observed in plants from all localities.

Four enzyme systems with seven loci (*Lap-1*, *Lap-2*, *6pgdh-1*, *6pgdh-2*, *Pgm-1*, *Pgm-2*, *Skdh*) were analyzed and allelicaly interpreted (Table 2). One system, EST, was interpreted as banding patterns only and results were compared with other systems.

Allozyme analysis revealed low genetic variation at both within- and between-population levels. Four loci (*Lap-1*, *6pgdh-1*, *6pgdh-2*, *Pgm-1*) proved to be monomorphic across all analyzed plants.

In total, four multilocus allozyme phenotypes were detected (Table 3). Three populations (Čenkov, Devín and Győr) were represented by phenotype I. The second, phenotype II, was discovered in the Balinka and Dorog populations. Two different, equally represented (genotypic eveness 0.96) phenotypes were found in the Pilis population (phenotypes III and IV); however, they differed at only one allozyme locus (*Lap-2*). Measures of allelic variation (mean number of alleles per locus, percentage of polymorphic loci, frequency of observed heterozygotes), and genotype diversity measures (D, E) for the studied populations are summarized in Table 3. High heterozygosity was found in all populations (observed heterozygosity ranged from 0.57 to 0.86).

Banding patterns of EST did not fully correspond with data from other enzyme systems. They did not reveal any within-population variation in the Pilis population. According to the EST banding patterns the populations of Balinka and Dorog possess different allozyme phenotypes. In the Devín, Čenkov and Győr populations, the EST banding pattern matches results from the other enzyme systems (one phenotype per allozyme only).

Discussion

Within-population variation

Genetic variation was low, especially at the within-population level. This fits well with the agamospermous mode of reproduction. However, in addition to the full (black) achenes some portion of empty (light brown to brown) ones were found in each experimentally emasculated head (17.0–35.1%). This may be explained by damage by cutting off the flower buds or by plant resource limitations. Conversely, the presence of facultative agamospermy and the versatility of the mode of reproduction and reproductive pathways (apospory, haploid parthenogenesis, cross-fertilization of both reduced and unreduced female gametes) in several species of *Hieracium* subgen. *Pilosella* (e.g. Krahulcová et al. 2004) may be an explanation. Thus, in some flowers reduced embryo sacs may have arisen and the ovule aborts due to a lack of sexual fusion in emasculated heads.

In respect to the wild populations, genetic uniformity most probably reflects the absence of sexual reproduction and predominance of (or even complete) agamospermy. If we consider that the potential for sexual reproduction (female meiosis) is present, genetic uniformity might be due to vegetative spread, e.g. by underground sprouts (a population that consists of one clone/genet and sexual reproduction is excluded due to self-incompati-

Table 2. – Allozyme phenotypes detected in six agamospermous populations of *Hieracium* *tauscheri* (a–d observed alleles). Banding patterns for EST were numbered and are presented as allozyme phenotypes (A-D) (allelic interpretation was not possible due to the complex banding pattern).

	Locality (number of plants)					
Locus	Balinka (4)	Čenkov (18)	Devín (13)	Dorog (18)	Győr (10)	Pilis (4+5)
Lap-1	aabb	aabb	aabb	aabb	aabb	aabb
Lap-2	cccc	bbcc	bbcc	cccc	bbcc	aacc, cccc
6pgdh-1	aaaa	aaaa	aaaa	aaaa	aaaa	aaaa
6pgdh-2	aabb	aabb	aabb	aabb	aabb	aabb
Pgm-1	aabb	aabb	aabb	aabb	aabb	aabb
Pgm-2	bbbb	bbcc	bbcc	bbbb	bbcc	bbdd
Skdh	bbcc	bccd	bccd	bbcc	bccd	abcc
EST	B	A	A	C	A	D

Table 3. – Measurements of allelic and genotypic diversity in agamospermous *Hieracium* *tauscheri.

Locality	Number of plants	Allozyme phenotype	Mean number of alleles per locus	Percentage of polymorphic loci	Observed heterozygozity	Number of multilocus genotypes	Genotypic diversity	Genotypic evenness
Čenkov	18	I	2	0	0.86	1	0	0
Devín	3	I	2	0	0.86	1	0	0
Győr	10	I	2	0	0.86	1	0	0
Balinka	4	II	1.57	0	0.57	1	0	0
Dorog	21	II	1.57	0	0.57	1	0	0
Pilis	4	III	1.86	14.29	0.79	2	0.55	0.96
	5	IV	1.86	14.29	0.79	2	0.55	0.96

bility). Self-incompatibilty has been found in many species of *Hieracium* subgen. *Pilosella*, incl. *H. echioides* (Kashin & Chernishova 1997, Peckert 2001). However, autogamy and the consequent decrease in genetic diversity can be caused by so called mentor effect (compatibility of own pollen grains induced by the presence of foreign ones). This has been documented in both subgen. *Pilosella* and subgen. *Hieracium* (Krahulcová et al. 1999, Mráz 2003).

Within-population variation was only found in the Pilis population. The detected variation is probably generated by hybridization with sexual *H. echioides* which was found in the neighbouring area. Sympatric occurrence of *H.* *tauscheri* and another species of subgen. *Pilosella* (*H. auriculoides* A. F. Láng, *H. bauhini – H. echioides*) was observed at the locality Dorog. Although plants of both taxa grow intermingled, no variation in *H.* *tauscheri* was detected. However, hybrid plants might morphologically resemble *H. auriculoides*, and therefore were not included in the allozyme analyses.

Between-population variation

All but one population (Pilis) were morphologically uniform. The Pilis population slightly differed in having larger flowering heads and looser inflorescences. Genetically all plants

from populations Čenkov, Devín and Győr belong to the same multilocus allozyme phenotype (phenotype I). The largest geographic distance between them is 123 km (Čenkov to Devín). The Dorog and Balinka populations (separated by 61 km), both possess phenotype II, with the exception of small differences in the EST banding patterns. The genetically differentiated population at Pilis is situated in area with nearly sympatrically occurring sexuals of *H. echioides,* and recent gene flow between the two cannot be excluded (see above).

It is hardly feasible to explain the origin of recent genetic variation in *H.***tauscheri* in the total distribution area. Studies of herbarium specimens in herbarium BP (Natural History Museum, Budapest) revealed rather common occurrences of various morphological types of *H. echioides* (s.l.) in the vicinity (or at least in the territory) of Budapest at the end of the 19th century and at the beginning of the 20th century. Among others, plants of var. *tauscheri* were revised. Most of them come from calcium rich eolic sands in the Danube Basin, and often from localities more or less influenced by human impact. Comparing past and present distributions, the number of localities has declined. Thus, recent geographically isolated and genetically identical populations (e.g. Devín, Győr and Čenkov) might be remnants of apomictic lines with more common distributions in the past. Populations at present survive on small refuges of continental grassland (e.g. Čenkov) or inhabit areas more or less influenced by human activities with disturbances and low competition. Similar to many other *Pilosella* taxa, *H.***tauscheri* does not persist in higher vegetation densities. On the other hand, detected agamospermous mode of reproduction might allow for rapid colonization of newly established open habitats.

Taxonomic position of Hieracium echioides subsp. echioides var. tauscheri

Hieracium subgen. *Pilosella* is characterized by taxonomical complexity which is caused mainly by the combination of frequent hybridization and the facultatively agamospermous mode of reproduction. A huge number of taxa have been described in this subgenus (Zahn 1921–1923). In *H. echioides*, seven subspecies are recognized (Zahn 1921–1923, 1922–1930); *H.***tauscheri* represents one of five varieties of *H. echioides* subsp. *echioides*. Based on our results, it is the only one hitherto known agamospermous taxon in the otherwise sexual *H. echioides*. Taxonomic revision of *H. echioides* and its related taxa is in progress. As we are aware of the taxonomic complexity of this group, we refrained now from taxonomic evaluation of our studied taxon and follow the nomenclature of Zahn's last monographic account (Zahn 1922–1930). Based on morphological characters, the investigated plants might represent an intermediate type derived from *H. echioides* and *H. piloselloides* Vill. It is possible that in addition to *H. piloselloides*, another hybridogeneous taxon might have participated in the introgression, most likely *H. auriculoides*. Further studies are needed to confirm the supposed origin of these plants.

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Souhrn

Populace agamospermicky se rozmnožujících rostlin *Hieracium echioides* byly objeveny na jižním Slovensku a v severním Maďarsku. Nalezené rostliny se od typického *H. echioides* liší nižší hustotou chlupů na lodyze a listech, měkčími chlupy, užšími a protáhlejšími listy a menšími úbory a odpovídají *H. echioides* subsp. *echioides* var. *tauscheri.* Cílem předkládané práce bylo zjistit genetickou strukturu, počet chromozomů a způsob rozmnožování u celkově 6 populací (Balinka, Čenkov, Devín, Dorog, Győr, Pilis). Bylo studováno 5 enzymových systémů (EST, 6PGDH, PGM, LAP a SKDH). Většina zymogramů byla interpretována alelicky, pouze v případě EST bylo pouze srovnáváno uspořádání proužků. Zjištěná genetická variabilita byla nízká na vnitro- i mezipopulační úrovni. Celkem byly nalezeny čtyři allozymové fenotypy (nebyly zohledněny výsledky EST). Tři populace (Čenkov, Devín, and Győr) se ukázaly být geneticky zcela shodné (fenotyp I), populace Dorog a Balinka byly reprezentovány rovněž jedním allozymovým fenotypem (fenotyp II), avšak systém EST ukázal, že každá populace je reprezentována odlišným fenotypem. Pouze v populaci rostlin z lokality Pilis byla nalezena vnitropopulační variabilita (fenotyp III a IV). Dva nalezené fenotypy se lišily v jednom lokusu a v populaci byly zastoupeny rovnoměrně. Karyologicky analyzované rostliny ze všech populací byly tetraploidní (2n = 36) a agamospermické (zjištěno metodou kastrace úborů); malá genetická variabilita dobře odpovídá zjištěnému způsobu rozmnožování. Vnitropopulační variabilita nalezená na lokalitě Pilis může být způsobena výměnou genů s blízkou populací sexuálně se rozmnožujícího *H. echioides*.Výskyt jednoho allozymového fenotypu na více lokalitách může odrážet širší a souvislejší rozšíření studovaných rostlin v minulosti v Podunají. Změny v krajině zapříčiněné činností člověka a tím způsobená ztráta vhodných lokalit vedly zřejmě ke zmenšení, rozdělení i zániku některých populací. Pro tuto hypotézu svědčí i data získaná studiem herbářového materiálu pocházejícího z přelomu 19. a 20. století.

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