

Rare recent natural hybridization in *Hieracium* s. str. – evidence from morphology, allozymes and chloroplast DNA

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Abstract. The first proven data on natural hybridization in the genus *Hieracium* s. str. are presented. Plants with intermediate morphological characters between the diploids *H. alpinum* and *H. transsilvanicum* were found in the Muntii Rodnei (Romanian Eastern Carpathians) in 2001 and in the Chornohora Mts (Ukrainian Eastern Carpathians) in 2003. While plants of intermediate morphology between usually so called basic species are usually tri- or tetraploid in *Hieracium* s. str., these plants were diploid ($2n=18$) like both parental species in this region. The Romanian plant did not produce fertile achenes in free pollination and in control backcrosses with *H. transsilvanicum*, two hybrids from Ukraine were completely seed sterile in free pollination and reciprocal crosses. Pollen stainability as an indirect measure of male fertility was quite high in the studied Ukrainian hybrid plants and similar to the parental taxa. Evidence from allozyme analysis also confirmed the hybrid origin of the studied plants. Sequencing and PCR-RFLP analyses of the *trnT-trnL* intergenic spacer revealed that all hybrid plants had the *H. transsilvanicum* chloroplast DNA haplotype. Maternal inheritance of chloroplast DNA in this particular cross was proved with artificial hybrids from reciprocal experimental crosses between *H. alpinum* and *H. transsilvanicum*. In both localities, the natural hybrid plants were found in disturbed habitats,

exceptionally allowing contact of the otherwise ecologically vicariate parental species. Morphologically, the hybrid plants belong to *H. xkrasani* Wol.

Key words: Asteraceae, chromosome numbers, hybrid zone, inheritance, allozymes, cpDNA, Carpathians

Introduction

The significance of interspecific hybridization in plant evolution has recently been stressed in many reviews (e.g. Arnold 1992, Rieseberg 1997, Rieseberg and Carney 1998). Consequently, documentation of natural hybridization is important for the description of evolutionary processes in particular taxa. Natural hybridization is unevenly distributed across taxonomic groups. Only ca 6–16% of the plant genera have one or more recorded hybrids (Ellstrand et al. 1996). Besides data on spontaneous hybridization, artificial crossing experiments have contributed much to a better understanding of microevolutionary processes like pre- and postzygotic isolation barriers, pollen competition, hybrid sterility, fertility

selection etc. (Rieseberg and Carney 1998, Lexer et al. 2003). Although morphological characters are used as a first step in the identification of hybrid plants in the field, they have limits, mainly in cases of introgression or in polyploid species complexes. Additional molecular approaches (allozymes, DNA analyses) have resulted in important insights in the role of hybridization and plant speciation in recent years (e.g. Bachmann 1994, Hedrén 1996, Morrell and Rieseberg 1998, Brochmann et al. 2000, Hedrén et al. 2000, Marhold et al. 2002, Nelson-Jones et al. 2002, Fehrer et al. 2005).

Hieracium L. s. str. belongs to the world's most species-rich plant genera (Royal Botanical Gardens Kew 1993). While most of the karyologically analyzed (micro)species are tri- or tetraploid with apomictic formation of seeds, diploid taxa are very rare and reproduce exclusively sexually, for some of them, strict allogamy has been proven (Rosenberg 1927; Bergman 1935, 1941; Battaglia 1947; Merxmüller 1975; Schuhwerk 1996; Chrtek 1997; Mráz 2003).

Hybridization probably plays a crucial role in the evolutionary processes in this genus, as revealed from the pattern of morphological characters. Many *Hieracium* species combine morphological characters of two or more basic species ("species principales", "Hauptarten") and are generally suggested to be of hybrid origin (described as "species intermediae", "Zwischenarten", cf. Zahn 1921–1923). They are apomictic polyploids like most of their putative parental species ("species principales", "Hauptarten"), and gene flow among them is severely restricted. With respect to the current predominant apomictic mode of reproduction, recent hybridization might only be expected between diploid sexual species (Merxmüller 1975) and between pollen producing polyploids and sexual diploids. Nevertheless, there are no reliable data on recent natural hybridization in the genus *Hieracium* s. str. so far. Only a few cases of spontaneous hybridization between cultivated plants have been recorded. Merxmüller (1975: 193) briefly noted

that a spontaneous hybrid arose from an interspecific cross between the diploids *H. porrifolium* L. and *H. umbellatum* L. cultivated in the Munich Botanical Garden. The supposed hybrid plants treated under the name "*H. leiocephalum*" Bartl. ex Griseb. had the same chromosome number ($2n = 18$) as its putative parental species according to labels of herbarium specimens deposited in Munich herbarium (M). However, there are no supplementary data that confirm true interspecific crosses (for this reason we do not use the special sign "×" to designate *H. leiocephalum* as a nothotaxon).

During field excursions in the Romanian (2001, P. Mráz) and the Ukrainian Eastern Carpathians (2003, P. Mráz and J. Chrtek), we found plants morphologically intermediate between *Hieracium alpinum* L. and *H. transsilvanicum* Heuff. Both species are diploid with sexual reproduction in this area (Pashuk 1987; Chrtek 1996, 1997; Mráz 2001, 2003; Mráz and Szeląg 2004). *H. alpinum* belongs to sect. *Alpina* (Griseb.) Gremler, and *H. transsilvanicum* to sect. *Vulgata* (Griseb.) Willk. & Lange (concept of sections follows Stace 1998), which are not morphologically closely related. Two taxa morphologically intermediate between *H. alpinum* and *H. transsilvanicum* have been described: *H. ×krasani* Woł. (according to Zahn either intermediate or closer to *H. transsilvanicum*) and *H. paxianum* Nyár. & Zahn (closer to *H. alpinum*). Both taxa are confined to the Eastern and Southern Carpathians (Zahn 1921–1923, 1930–1939; Nyárády 1965). They were, similarly to other "intermediate species" of *Hieracium*, originally supposed to be of hybridogenous origin (e.g. Zahn 1921–1923, 1930–1939), and have sometimes also been treated as hybrid taxa (nothospecies) (in the case of *H. ×krasani*; Wołoszczak 1890: 65, wrote the phrase "inter parentes", from which it may be suggested that Wołoszczak himself considered the newly described taxon to be a true hybrid, see Discussion). However, their hybrid origin has never been confirmed experimentally. More recently, it became a general notion that recent hybridization in *Hieracium* s. str. could be largely discounted because of

Table 1. Origin of plant material and analyses performed

Taxon	Cultivation no.	Locality	Allozyme analysis	PCR-RFLP	Sequencing (GenBank)	Pollen viability	Chromosome numbers
<i>H. alpinum</i>	JC H 866/ 1–10	Ukraine, Chornohora Mts, Polonina Breskulska ridge, the saddle between Mt. Hoverla and Mt. Breskul, 1800 m a.s.l., 48°09'09.8" N, 24°30'14.6", coll. P. Mráz & J. Chrtek jun., 23 July 2003	+	+		+	+
	PM Rodna2	Romania, Munții Rodnei Mts, glacial cirque on the NE slopes of Mt. Pietrosul Mare, ca 0.3 km SE from Stația Meteo, ca 1900 m a.s.l., coll. P. Mráz, 5 July 2001, 'alp.Boa.2' (sequence identical to 'alp.Ukr')			+		
	HERB JC (PRA)	Ukraine, Chornohora Mts, Mt. Pozhizhevska, SW slope, ca 1780 m a.s.l., coll. J. Chrtek jun., J. Hadinec & J. Michálek, 22 July 1994 'alp.Ukr'			+		+
<i>H. transsilvanicum</i>	JC H 864/ 1–12	Ukraine, Chornohora Mts, Polonina Breskulska ridge, at tourist path from the village of Hoverla to Mt. Hoverla, 1410 m a.s.l., 48°08'35.0" N, 24°28'56.7", coll. P. Mráz & J. Chrtek jun., 23 July 2003	+	+		+	+
	PM 1066	Romania, Munții Rodnei Mts, border of the tourist path from the village of Borșa to Mt. Pietrosul Mare, spruce forest, 1300–1400 m a.s.l., 47°39' N, 24°39' E, coll. P. Mráz, 5 July 2001, 'tra.Boa'			+		+

Table 1. Origin of plant material and analyses performed

Taxon	Cultivation no.	Locality	Allozyme analysis	PCR-RFLP	Sequencing (GenBank)	Pollen viability	Chromosome numbers
<i>H. alpinum</i> × <i>H. transsilvanicum</i> = <i>H. xkrasani</i> (natural hybrids)	JC H 863, PM 1399, PM 1400	Ukraine, Chornohora Mts, Polonina Breskulska ridge, at tourist path from the village of Hoverla to Mt. Hoverla, 1410 m a.s.l., 48°08'35.0" N, 24°28'56.7", coll. P. Mráz & J. Chrtek jun., 23 July 2003	+	+	+	+	+
	PM 985	Romania, Munții Rodnei Mts, border of the tourist path from the village of Bor ^o a to Mt. Pietrosul Mare, spruce forest, 1350 m a.s.l., 47°39' N, 24°39" E, coll. P. Mráz, 5 July 2001		+			+
<i>H. alpinum</i> × <i>H.</i> <i>transsilvanicum</i> (experimental hybrids)	PM X5/5, X5/6	mother plant: <i>H. alpinum</i> no. 649, pollen donor: <i>H. transsilvanicum</i> no. 1064		+			+
	PM X2/16	mother plant: <i>H. transsilvanicum</i> no. 1064, pollen donor <i>H. alpinum</i> no. 649 (for localities of both parents see Mráz 2003)		+			+

the common apomictic mode of reproduction in completely prevailing polyploid taxa (Merxmüller 1975).

The main aim of the present study was to test the hypothesis about the recent hybrid origin of the morphologically intermediate plants collected by us in the Eastern Carpathians. We performed a set of analyses including detailed morphological observations, chromosome counting, pollen fertility estimation, observation of seed production in free pollination and control hybridization, allozyme analyses, cpDNA sequencing and PCR-RFLP analyses.

Material and methods

Plants. The supposed hybrid plants from Romania and Ukraine, as well as plants of the putative parental species (*Hieracium alpinum* and *H. transsilvanicum*) from the same localities were cultivated in the experimental field of the Botanical Garden of the P. J. Šafárik University in Košice and in a glasshouse at the Institute of Botany, Academy of Sciences of the Czech Republic in Průhonice. Voucher specimens are deposited in the Herbarium P. Mráz in Košice (plants marked as PM) and at the Institute of Botany, Průhonice (PRA, plants marked as JC). Additionally, three diploid artificial hybrids from reciprocal control crosses between *H. alpinum* and *H. transsilvanicum* were included in our study to test the maternal inheritance of cpDNA (cf. Mráz 2003). Details about all plants studied are given in Table 1.

Chromosome numbers and breeding system. All cultivated plants subsequently used for molecular studies were checked for their chromosome number. Two different methods were used: (i) root tip cuttings were pretreated with 0.5% solution of colchicine for 1.5–3 hours at room temperature, subsequently fixative (ethanol and glacial acetic acid, 3:1) replaced colchicine, roots were stored in 70% ethanol and hydrolyzed for 5–7 minutes in 1N HCl at 60°C. The squash and smear method with cellophane replacing the glass covers followed Murin (1960). Giemsa solution in phosphate buffer was used for staining (method used by P. M.); (ii) actively growing roots were placed in a pretreatment solution of saturated p-dichlorobenzene for 3–4 hours, then 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; method used by J. C.). Altogether, chromosome numbers for 31 plants were determined (for details see Table 1).

In order to determine the breeding system of the putative hybrid plants, one capitulum of PM 985 was crossed with one of the putative parents, *Hieracium transsilvanicum* (PM 1064), which was flowering at the same time as the hybrid; and two capitula of supposed hybrids PM 1399 and PM 1400 were crossed reciprocally. The inflorescences used in control crosses were isolated by nylon bags until anthesis. Another capitulum of PM 985 and two other capitula of PM 1399 and 1400 were kept unisolated for free pollination.

Male fertility was estimated as pollen stainability using the acetocarmin method in glycerol jelly (Marks 1954). Three unopened tubular flowers per capitulum were removed and carefully cut with a razor blade in one drop of acetocarmine jelly in order to remove the pollen grains. 100–150 grains were evaluated per individual and both viable (well-stained) and non-viable (unstained) grains were scored. Two putative hybrid plants and three individual plants of both putative parental species from the Ukrainian locality were studied in this way. Because all capitula of the putative hybrid plant from Romania (PM 985) were included in the control crosses and free pollination (see above), this plant was not studied for pollen stainability.

Allozyme analyses. A total of 25 diploid plants from the Ukrainian locality were used for the analysis, i.e. 10 plants of *Hieracium alpinum* (JC H 866), 12 plants of *H. transsilvanicum* (JC H 864) and 3 plants of presumed hybrid origin (JC H 863, PM 1399 and PM 1400). Extraction, electrophoresis and staining followed the methods described in Štorchová et al. (2002). The following enzyme systems were examined: AAT (Aspartate aminotransferase, EC 2.6.1.1, dimeric), ADH (Alcohol dehydrogenase, EC 1.1.1.1, dimeric), LAP (Leucine aminopeptidase, EC 3.4.11.1, monomeric), PGM (Phosphoglucomutase, EC 5.4.2.2, monomeric), 6PGD (6-phosphogluconate dehydrogenase, EC 1.1.1.44, dimeric), and SKD (Shikimic acid dehydrogenase, EC 1.1.1.25, monomeric). The average number of alleles per locus, percentage of polymorphic loci, observed and expected heterozygosity (Levene 1949), Shannon's diversity index, and the number of different multilocus genotypes were

calculated for *H. alpinum* and *H. transsilvanicum*, respectively. A primary data matrix based on presence / absence of alleles of eight allelically interpretable loci (see Table 2) for each studied plant was analysed by principal coordinate analysis (PCoA) using Jaccard's coefficient (SYN-TAX 2000, Podani 2001).

Chloroplast DNA analyses. DNA isolations were done as described by Štorchová et al. (2000), but fresh, silica gel-dried or herbarium material was used. In order to determine the maternal inheritance in artificial progeny from reciprocal crosses between two putative parental species and the maternal parent of the natural hybrids, a PCR-RFLP approach was designed based on a larger data set from the *trnT-trnL* intergenic spacer of

Hieracium chloroplast DNA (Fehrer et al. 2005; and unpublished data). According to this, the parental taxa *H. alpinum* and *H. transsilvanicum* belong to slightly divergent cpDNA lineages discriminated by four substitutions and three small indels. One of the substitutions resulted in a loss of an *EcoR* I restriction site in *H. transsilvanicum* that was unique for the whole genus. One *H. transsilvanicum* plant from Romania and two *H. alpinum* plants were sequenced: a diploid one from the Ukraine (herbarium material) and one from a locality close to the Romanian hybrid population. The *H. alpinum* sequences were identical. One sequence per species was deposited in the GenBank database (accession numbers AY512556–AY512557). The hybrids' chloroplast haplotype

Table 2. Morphological characters of parental species (Zahn 1930–1939, Chrtek 1997, additional own observations include 20 plants per species) and hybrid plants (own observations on 4 plants)

Character	<i>H. transsilvanicum</i>	<i>H. ×krasani</i>	<i>H. alpinum</i>
Height of plant (m)	(0.2–)0.3–0.6(–0.8)	0.25–0.40	0.05–0.2(–0.3)
No. of stem leaves	(1–)2–5	2–3	0–1(–4)
No. of heads	(3–)5–20	3–6	1
Branching	upper third / quarter of stem	usually upper third of stem	stem unbranched
Length of involucre bracts (mm)	7–9	10–11	12–15
Width of involucre bracts (mm)	0.7–0.9	0.8–1.0	0.9–1.2
Glandular trichomes on the leaves	absent	scattered	scattered to numerous
Simple eglandular trichomes on peduncles and involucre bracts	absent	scattered to numerous	scattered to numerous
Stellate trichomes on involucre bracts	rare to scattered on the base and margins	rare on the base and sometimes on the margins	absent
Colour of simple eglandular trichomes of rosette leaves	reddish	white to reddish	white
Simple eglandular trichomes at the apex of ligules	absent	absent	scattered to numerous
Colour of styles	dark yellow to brown often with black scales	brown with black scales	yellow to dark yellow

was determined by PCR-RFLP. The *Eco*R I-digested fragments were separated on 3% agarose gels. PCR, RFLPs and sequencing were done as described previously (Fehrer et al. 2005).

Results

Morphology. The putative hybrids are intermediate between *Hieracium alpinum* and *H. transsilvanicum* (Fig. 1). The main distinguishing characters concern branching pattern, number of heads and indumentum of the peduncles and involucre bracts (details given in Table 2).

Chromosome numbers and breeding system. Chromosome numbers were determined for 31 plants including the four natural hybrids, three artificial hybrids from reciprocal crosses and both parental species (Table 1). For all of them, $2n = 2x = 18$ were counted (Fig. 2).

A pollen stainability of 78% and 96% was observed in the analyzed Ukrainian hybrid plants (PM 1400 and JC 863 respectively), while pollen stainability ranged from 78–93% in *Hieracium alpinum*, and from 91–99% in *H. transsilvanicum*. This suggests the hybrids have similar male fertility to both parental taxa.

In the Romanian hybrid plant (PM 985), no well-developed achenes were produced in either free pollination or in a control backcross with *Hieracium transsilvanicum*. The same results we obtained from control reciprocal crosses between the hybrid plants from Ukraine (PM 1399 and 1400) and from free pollination of both plants. All three tested putative hybrid individuals were completely seed sterile.

Allozyme analyses. Six enzyme systems with 11 loci were investigated in *Hieracium*



Fig 1. A *Hieracium alpinum* (Herb. P. Mráz s.n.), B natural hybrid plant of *H. xkrasani* (Herb. PRA s.n.) and C *H. transsilvanicum* (Herb. PRA s.n.), scale bar = 5 cm

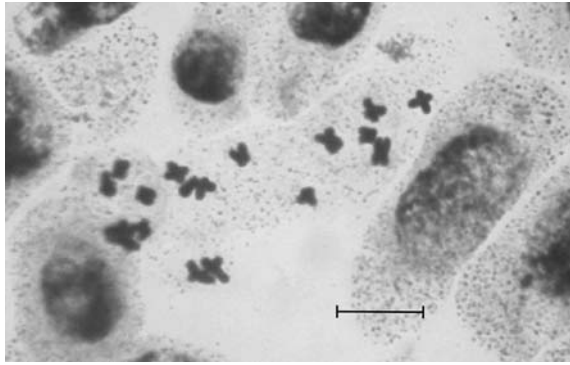


Fig 2. Chromosomes ($2n = 18$) in a root tip cell of the natural hybrid *H. xkrasani* (PM 1399), scale bar = 10 μm

alpinum, *H. transsilvanicum* and their putative hybrids. Two loci were excluded from further analyses: *Aat-1* (it turned out to be monomorphic in all studied plants) and *Pgm-2* (low enzyme activity). Altogether nine loci were therefore evaluated. Genotype frequencies of each locus are given in Table 3.

Hieracium alpinum and *H. transsilvanicum* differed consistently in five loci. The hybrid plants exhibited a unique additive genotype for *Skd*. For another two loci, they shared the genotype with *H. alpinum*, for three loci with *H. transsilvanicum*, and for three loci with both parents. Contribution of *H. alpinum* was con-

firmed in patterns for *Pgm-1* (allele c) and *Lap-1*. While bands of *Lap-1* are consistently lacking in *H. transsilvanicum* (besides the present plants, the same results were obtained for other *H. transsilvanicum* plants studied in 1999, Chrtek unpubl. data), the hybrid plants share these alleles with *H. alpinum*. For *H. alpinum* and *H. transsilvanicum*, the mean number of alleles per locus, percentage of polymorphic loci, observed and expected heterozygosity, number of multilocus genotypes, mean genotype diversity within loci and Shannon's diversity index are summarized in Table 4. The level of allelic variation is higher in *Hieracium transsilvanicum* than in *H. alpinum*.

The three hybrid plants shared the same multilocus allozyme phenotype. PCoA placed them between the parents along the first coordinate (Fig. 3). Allozyme analyses clearly show genetic contributions of both parental species indicating true hybrid origin.

Chloroplast DNA analyses. The Ukrainian *Hieracium transsilvanicum* was characterized by the same unique loss of an *EcoR* I restriction site as the Romanian sample of *H. transsilvanicum* from the second hybrid locality used for sequencing, allowing the distinction of the parental haplotypes by PCR-RFLP. The

Table 3. Allozyme genotypes and their frequencies. For *Adh-2*, only banding patterns were compared (not interpretable genetically). Bands of *Lap-1* were not present in *H. transsilvanicum*

Taxon (number of plants)	Locus and genotype frequencies								
	<i>Aat-2</i>	<i>Adh-1</i>	<i>Adh-2</i>	<i>Lap-1</i>	<i>Lap-2</i>	<i>Pgm-1</i>	<i>6Pgdh-1</i>	<i>6Pgdh-2</i>	<i>Skd</i>
<i>H. alpinum</i> (10)	bb 1.00	ab 0.10	7 0.20	ab 0.10	aa 0.40	ac 1.00	aa 1.00	bb 1.00	aa 1.00
		bb 0.90	8 0.40	bb 0.60	ab 0.60				
			9 0.30	bc 0.20					
			10 0.10	cc 0.10					
<i>H. xkrasani</i> (3)	bb 1.00	bb 1.00	2 1.00	bb1.00	ab 1.00	ac 1.00	bb 1.00	ab 1.00	ac 1.00
<i>H. transsilvanicum</i> (12)	aa 0.08	bb 1.00	1 0.08	–	aa 0.92	ab 1.00	bb 1.00	aa 0.08	bb 0.25
	ab 0.58		2 0.50		ab 0.08			ab 0.34	bc 0.42
	bb 0.34		3 0.08					bb 0.58	cc 0.33
			4 0.08						
			5 0.08						
		6 0.18							

Table 4. Measures of allelic and genotypic variation. N = number of individuals; A = mean number of alleles per locus; P = percentage of polymorphic loci; H_o = observed heterozygosity, H_{exp} = expected heterozygosity (computed according to Levene 1949); I = Shannon's index, G = number of different multilocus genotypes

Species	N	A	P	H_o	H_{exp}	I	G
<i>H. alpinum</i>	10	1.625	50.00	0.250	0.176	0.274	7
<i>H. transsilvanicum</i>	12	1.714	71.43	0.345	0.286	0.397	10

maternal inheritance of the plastome in *Hieracium* s. str. was confirmed by PCR-RFLP for the particular combination of taxa by examination of three artificial hybrids from reciprocal crosses between *H. transsilvanicum* and *H. alpinum*. The natural hybrids from both, Romanian and Ukrainian, populations showed the chloroplast haplotype of *H. transsilvanicum* (Fig. 4) indicating that this species was the seed parent in all cases. Attempts to do molecular analyses with the syntype specimen of *H. ×krasani* (see below) were unsuccessful as it did not contain usable DNA because of its age (collected in 1888).

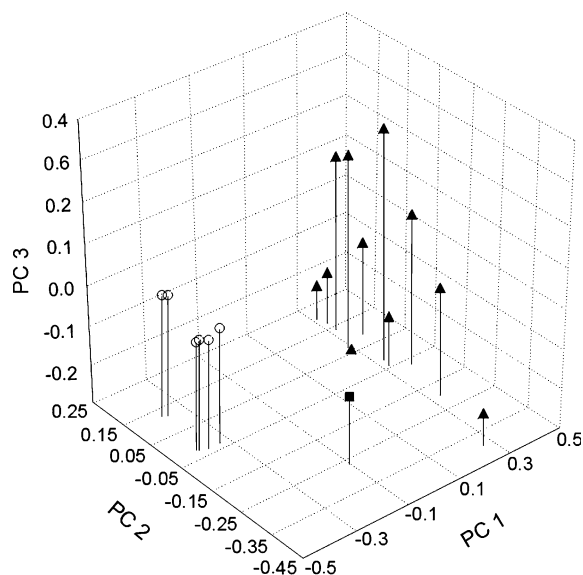


Fig 3. Principal coordinate analysis of allozyme data of *Hieracium alpinum* (circles), *H. transsilvanicum* (triangles) and hybrid plants (square)

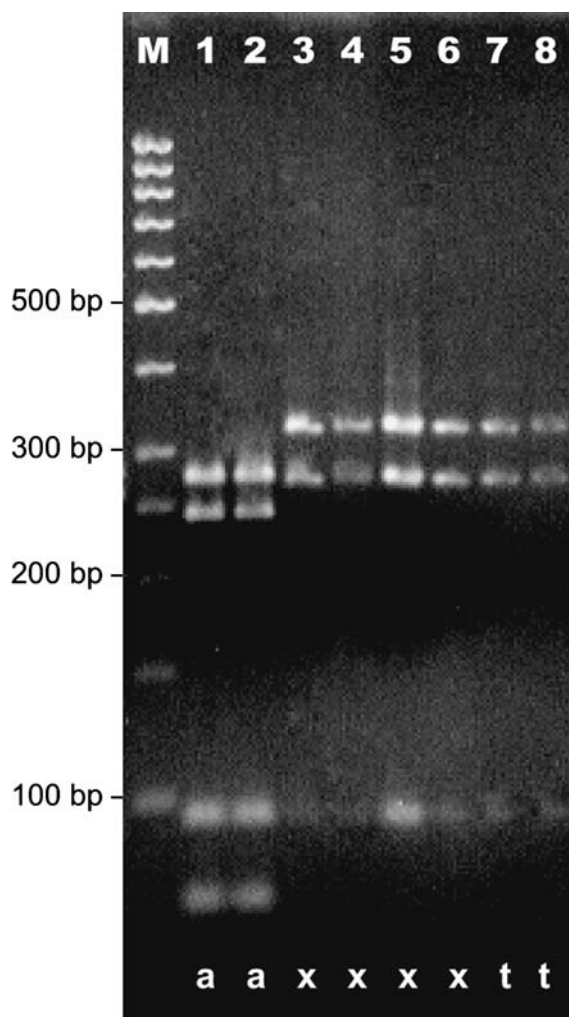


Fig 4. PCR-RFLP of the *trnT-trnL* intergenic spacer of chloroplast DNA. M: marker; 1 and 2: *H. alpinum* ('a'); 3–6: hybrid plants ('x'); 7–8: *H. transsilvanicum* ('t'). The hybrid in track 4 was from Romania, the three others were from the Ukrainian population

Discussion

Hybrid origin of the plants. The investigated diploid hybrid plants *Hieracium alpinum* × *H. transsilvanicum* showed a high degree of morphological intermediacy between both parental species. However, they have slightly more characters in common with the maternal parent, *H. transsilvanicum*. Similar results have been obtained from artificial reciprocal crosses between the same diploid taxa, the hybrid progeny more closely resembled the maternal plants in both directions of the cross (Paule 2004). All karyologically studied plants (*H. alpinum*, *H. transsilvanicum* and the hybrid plants from nature as well as from artificial hybridization) were shown to be diploid. Our counts are in agreement with previously published chromosome numbers in both *H. alpinum* and *H. transsilvanicum* from the Eastern Carpathians (Pashuk 1987; Chrtek 1996, 1997; Mráz 2001, 2003; Mráz and Szelağ 2004). Further hybrids from experimental reciprocal crosses were also diploid (Paule 2004). The Ukrainian hybrids produced a considerable quantity of homogeneously sized pollen with comparable stainability to the parental species, indicating regular microsporogenesis. All studied hybrid plants only produced completely empty achenes in free pollination as well as in control experimental crosses. Thus, female sexual reproduction was limited or even excluded in the hybrid plants. This finding is similar to what has been found in artificial *Hieracium* hybrids from several different diploid – diploid crosses. Megasporogenesis in artificial diploid hybrids was usually highly disturbed, and production of normally developed achenes in free and control pollination was either absent or very low (in the range of 0–12.1%) (Mráz and Paule 2003, Paule 2004, Mráz and Paule, unpubl. data). On the other hand, the hybrids might contribute to sexual reproduction as pollen donors. The artificial hybrids generally produced a rather large quantity of stainable pollen grains of homogeneous size, similar to our natural hybrid and its parents (Mráz and Paule 2003, Paule 2004, Mráz and Paule,

unpubl.). Vegetative spread by means of short rhizomes is of little importance in *Hieracium* s.str. Nevertheless, just the hybrid plants from the Ukraine are probably results of such vegetative spread (they grew side by side in a spot of ca 30 × 30 cm and shared the same multilocus allozyme phenotype).

Allozyme data also supported the hybrid origin. The hybrids did not show any unique alleles, but rather exhibited an additive pattern for *Skd*; for the remaining polymorphic loci, the hybrids shared its single-locus phenotypes with either *Hieracium alpinum* or *H. transsilvanicum*. Contribution of *H. alpinum* was clearly confirmed in patterns from two loci (*Pgm-1*, *Lap-1*). Intrapopulation genetic variation of *H. alpinum* and *H. transsilvanicum* reflected their sexual mode of reproduction in the study area.

Frequency of hybridization. Recent spontaneous hybridization between *Hieracium alpinum* and *H. transsilvanicum* is undoubtedly a very rare event. Only a few plants (which are the subject of the present paper) were found during our excursions to the Ukrainian and Romanian Eastern Carpathians. This matches the very low number of what is presumably the same hybrid taxon preserved in public herbarium collections. To explain the rarity of hybridization events in *Hieracium* s.str. and between *H. alpinum* and *H. transsilvanicum* in particular, both internal and external reproduction barriers should be considered.

The parental species belong to different sections within the genus *Hieracium*, and are not morphologically closely related. Thus, chromosomal and genic incompatibilities might play an important role. Another feature that may contribute to the generally low rate of hybridization is a mentor effect, i.e. induction of self-compatibility in otherwise incompatible pollen when it is mixed with foreign pollen (Richards 1997: 223), which was recently discovered during control crosses between several diploid as well as between diploid and polyploid taxa (Mráz 2003). In many different crosses the proportion of autogamously derived progeny from diploid

mother plants reached 100%, although the diploid species are usually strictly self-incompatible (Chrtek 1997, Mráz 2003). Thus, the mentor effect may represent a very effective hybridization barrier in this diplosporic genus. In the closely related genus *Pilosella* (often treated as a subgenus of *Hieracium*), autogamy could also be stimulated by pollen from other species under certain conditions (Krahulcová et al. 1999), but its degree is substantially lower and its role in the generally self-incompatible aposporic *Pilosella* is very limited as reflected by the vast amount of recent hybridization (Krahulcová et al. 2000). A high level of selfing (90%) induced by the influence of foreign pollen was also recorded in crosses between sexual diploid (mother plants) and apomictic triploid (pollen donors) dandelions (*Taraxacum* Wigg.; Tas and van Dijk 1999). Apart from induced autogamy by heterospecific pollen, pollen competition should be taken into account. Studies on *Iris* L. and *Helianthus* L. showed the advantage of conspecific against heterospecific pollination (Carney et al. 1994, Rieseberg et al. 1995, Emms et al. 1996, Carney and Arnold 1997).

Besides internal barriers, there are some external ones preventing natural hybridization between *Hieracium alpinum* and *H. transsilvanicum*. Among the factors that are considered most critical to rates of hybridization are differences in ecological preferences. While *H. transsilvanicum* is a typical element of spruce (and fir-beech) forests of the Eastern and Southern Carpathians, *H. alpinum* is restricted to the grassland of the alpine and, extremely rarely, subalpine belts. Both taxa are clearly separated by altitudinal and ecological demands (e.g. light intensity). At the Romanian locality a single hybrid plant was found on a tourist path at 1350 m altitude in the spruce belt. It was accompanied by plants of both parental species. While *H. transsilvanicum* was abundant in its typical biotope, *H. alpinum* occurred with three individuals only ca 300 m from the hybrid site. The presence of *H. alpinum* at this place may be explained by the close proximity of the alpine belt and by the fact

that the locality was strongly disturbed (and cleared) by forest machines during cutting. The biotope is thus suitable for incidental occurrence of *H. alpinum* at an atypical altitude. The small group of hybrid individuals (altogether 5 flowering plants) from the Ukraine – representing probably a single clone of plants arisen via vegetative propagation of rhizomes – grew at the border between spruce forest and secondary pasture at 1410 m altitude. As in the first case, *H. transsilvanicum* was abundant, but *H. alpinum* as pollen donor was completely absent from the locality. The next closest plants of this arcto-alpine species were observed along the disturbed margins of a tourist path at an altitude of 1600 m, ca 0.7–1 km away. In both cases, the hybrid plants were found at intermediate altitudes in biotopes disturbed by human activity, where both parental species came into (more or less close) secondary contact.

The importance of habitat disturbance providing the corridors for species movement and leading to sympatry in otherwise allopatric species as a prerequisite for hybridization was stressed e.g. by Levin et al. (1996). Similar secondary contacts may have taken place during the major climatic changes of the Pleistocene glaciation and in the short post-glacial period. During this period of advancement and retreat of glaciers, new types of biotopes arose, where sympatry of many different species and thus interspecific hybridization became possible (Asker and Jerling 1992, Carman 2001). The morphological variability in the genus *Hieracium* likely reflects an immense reticulate evolution in the past. While external barriers are not obvious among all diploids found in this genus (at present, a total of no more than 25 known diploid taxa, cf. Chrtek et al. 2004), most of them are separated ecologically and/or geographically from each other. The great majority of diploid taxa belongs to the “basic” species in the sense of Zahn (1921–1923). As in the genus *Hieracium* s. str. where there are many (micro)species with intermediate morphology between different “basic” species

(which are also considered as putative parents and often have different ecological demands, however), we can assume that new hybridogenous taxa may typically arise in hybrid zones of secondary contact. Because most of the counted taxa are polyploids, hybridization processes in *Hieracium* had to be followed by a rise in ploidy level and by apomictic reproduction (but not in the case of our hybrids!) as an “escape from sterility”. Hybridization in polyploid *Hieracium* species is strongly limited by their mode of reproduction. Agamospermy with full omission of female meiosis (diplospory of *Antennaria* type) has been recorded, followed by autonomous development of endosperm (e.g. Rosenberg 1927, Bergman 1941, Gustafsson 1946). Of crucial importance is precocious embryony – the unreduced egg cell develops into the embryo before the flower opens so that fertilization is impossible (Nogler 1984). Thus, the possibility of hybrid formation with *Hieracium* polyploids serving as mother plants is highly limited. Gene flow is more likely either among diploid taxa (as presented in this paper), or between diploids (as mother plants) and pollen-producing polyploids (as pollen donors).

Direction of hybridization. Apart from a few exceptions, maternal inheritance of chloroplast genes is typical in angiosperms (reviewed in Birky 1995, 2001) and was also shown recently for the closely related genus *Pilosella* (Fehrer et al. 2005). Analysis of artificial hybrid progeny from reciprocal hybridization between *Hieracium alpinum* and *H. transsilvanicum* with chloroplast DNA markers confirmed that the plastome is indeed inherited maternally in the cross in question. As the natural hybrids from Romania and Ukraine shared their chloroplast haplotype with *H. transsilvanicum*, this species apparently served as maternal parent in all cases. Recently, Mráz and Paule (2003) and Paule (2004) made a series of experimental crosses between selected diploid sexual species and between diploid sexual (mother plants) and polyploid apomictic (pollen donors) species. Most of the

F1 plants from diploid – diploid crosses proved to be true hybrids. In contrast, reciprocal crosses between *H. alpinum* and *H. transsilvanicum* seemed to depend somewhat upon the direction of the cross: While in the cross with *H. transsilvanicum* as seed parent all ten progeny plants were true hybrids, in the cross with *H. alpinum* as maternal plant, four out of 21 plants were of autogamous origin (Mráz 2003). Higher susceptibility for induced autogamy (mentor effect) in *H. alpinum* than in *H. transsilvanicum* might partially explain that the latter taxon served as maternal parent in both cases of natural hybrids. However, as this assumption is based on a single reciprocal cross only, more experimental data would be needed to draw firm conclusions about this aspect.

Initially, we assumed that *H. alpinum* was the much likelier seed parent for two reasons. (i) Due to the abundance of *H. transsilvanicum* and the absence or rarity of *H. alpinum* at both sites, a single or a few *H. alpinum* plants incidentally occurring outside their natural habitat were expected to be exposed to an excess of *H. transsilvanicum* pollen. (ii) While the plants of both parental species grew close to each other at the Romanian locality, at the Ukrainian locality the parental species were separated by a distance of at least 700 m. Pollinators are usually not expected to exceed about 30 m of flying distance (e.g. Proctor et al. 1996, Richards 1997) whereas *Hieracium* seed dispersal might be rather efficient due to possession of a hairy pappus. Therefore the result from chloroplast DNA analysis that *H. transsilvanicum* acted as seed parent was rather surprising, especially in the case of the Ukrainian hybrids. Possible explanations are: (i) individual plants of *H. alpinum* in closer vicinity to the hybrids might have been overlooked, (ii) pollinators bridged a longer distance in this case, (iii) the hybrids were present at the site for some time already and previously occurring *H. alpinum* meanwhile disappeared as community parameters do not allow its long-term survival, or (iv) the hybrids could have arisen at another locality where both parental taxa grew in closer contact and the

achenes were later wind-dispersed to where the plants were found.

Another possibility is that our hybrids already represent a backcross to *H. transsilvanicum*. In such case, the hybrid would – independent from the original direction of the cross – have acted as pollen parent (as it was highly probably seed sterile, too) so that the BC1 would, in any case, display *H. transsilvanicum* cpDNA. As F1 plants are highly heterozygous due to the pronounced differences between the parental species, products of a BC1 should be variable due to segregation and morphologically fill the whole space between the parents. It is therefore hardly possible to distinguish the result of a BC1 from a true F1. Our hybrids' closer resemblance to *H. transsilvanicum* also would not contradict the backcross scenario.

Taxonomic status of the hybrid plants.

Morphologically, the studied hybrid plants belong to *Hieracium* \times *krasani* Woł. (Wołoszczak 1890, as “*H. Krašani*”). This hybrid was described from two localities (Mt. Siniak and Mt. Kukul) in the Chornohora Mts. in the Ukrainian Eastern Carpathians, where it was collected in stands of *H. alpinum* and *H. transsilvanicum* (see below). We found one herbarium specimen (syntype) corresponding to the protologue. The label reads as follows: “*Hieracium alpinum* \times *pleiophyllum* (*H. Krašani* Woł.). In alpe Siniak (pr. Tatarów) Carpathorum orientaliu Galiciae, ca 1600 m sm., 8/1888, legit Wołoszczak” (LWS s.n.). It is not clear if Wołoszczak himself considered *H. \times krasani* as a true hybrid of recent origin or a hybridogenous taxon. The first hypothesis is favoured by his words in the protologue “*H. Krašani* (*H. alpino* \times *transsilvanicum*) ... inter parentes absque aliis *Hieraciis* rarissimum ...” (cf. Wołoszczak 1890: 65). On the other hand, the “ \times ” sign (or others like \leq , \geq , $<$, $>$; e.g. Zahn 1921–1923) are used as a short description of the morphological position of particular intermediate taxa between two or more “basic” species in the genus *Hieracium*. Moreover, the distinction between a hybrid taxon (nothotaxon) and a hybridogenous one was

not clear at that time. In our opinion, the hybrid plants collected in Romania and Ukraine are clearly of recent origin. The plants were found at the localities as a single or only a few individuals, they are seed sterile, and they have the same diploid chromosome number as their parental species. We therefore treat them in the present paper as nothotaxon in the sense of the International Code of Botanical Nomenclature (Greuter et al. 2000). Wołoszczak also reported *H. (\times) krasani* as a rare (notho)taxon from two original localities (see above), later on he published one additional record of just one plant from another locality of the Ukrainian Eastern Carpathians (Wołoszczak 1894: 143, “... w dziedzinie kosodrzewu w jednym okazie na Popadi” [the only one plant was found in the subalpine belt on Mt. Popadia]). In contrast, most of Zahn’s “species intermediae” are actually polyploids with apomictic breeding systems and they are treated as separate taxa, i.e., not as hybrids. They usually occur in high individual numbers, and they are considered to be of hybridogenous origin.

A similar taxon of intermediate habit between *H. alpinum* and *H. transsilvanicum* from the Southern Carpathians (Munții Retezatului, Romania) was found to be triploid ($2n = 27$) (Mráz unpubl.). However, this triploid species is morphologically different (in the density of indumentum, and partially in the shape of rosette leaves) from the hybrids of *H. \times krasani* reported in our study.

Probably *H. \times krasani* is the first true nothotaxon in the genus *Hieracium* s. str. to be verified by a set of different experimental approaches.

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