



Genetic structure of *Hypochaeris uniflora* (Asteraceae) suggests vicariance in the Carpathians and rapid post-glacial colonization of the Alps from an eastern Alpine refugium

Patrik Mráz*, Myriam Gaudeul†, Delphine Rioux, Ludovic Gielly, Philippe Choler, Pierre Taberlet and the IntraBioDiv Consortium‡

Laboratoire d'Ecologie Alpine, CNRS UMR 5553, Université Joseph Fourier, PO Box 53, F-38041 Grenoble Cedex 9, France

ABSTRACT

Aim The range of the subalpine species *Hypochaeris uniflora* covers the Alps, Carpathians and Sudetes Mountains. Whilst the genetic structure and post-glacial history of many high-mountain plant taxa of the Alps is relatively well documented, the Carpathian populations have often been neglected in phylogeographical studies. The aim of the present study is to compare the genetic variation of the species in two major European mountain systems – the Alps and the Carpathians.

Location Alps and Carpathians.

Methods The genetic variation of 77 populations, each consisting of three plants, was studied using amplified fragment length polymorphism (AFLP).

Results Neighbour joining and principal coordinate analyses revealed three well-supported phylogeographical groups of populations corresponding to three disjunct geographical regions – the Alps and the western and south-eastern Carpathians. Moreover, two further clusters could be distinguished within the latter mountain range, one consisting of populations from the eastern Carpathians and the second consisting of populations from the southern Carpathians. Populations from the Apuseni Mountains had an intermediate position between the eastern and southern Carpathians. The genetic clustering of populations into four groups was also supported by an analysis of molecular variance, which showed that most genetic variation (almost 46%) was found among these four groups. By far the highest within-population variation was found in the eastern Carpathians, followed by populations from the southern and western Carpathians. Generally, the populations from the Alps were considerably less variable and displayed substantially fewer region-diagnostic markers than those from the south-eastern Carpathians. Although no clear geographical structure was found within the Alps, based on neighbour joining or principal coordinate analyses, some trends were obvious: populations from the easternmost part were genetically more variable and, together with those from the south-western part, exhibited a higher proportion of rare AFLP fragments than populations in other areas. Moreover, the total number of AFLP fragments per population, the percentage of polymorphic loci and the proportion of rare AFLP fragments significantly decreased from east to west.

Main conclusions Deep infraspecific phylogeographical gaps between the populations from the Alps and the western and south-eastern Carpathians suggest the survival of *H. uniflora* in three separate refugia during the last glaciation. Our AFLP data provide molecular evidence for a long-term geographical disjunction between the eastern and western Carpathians, previously suggested from the

*Correspondence: Patrik Mráz, Laboratoire d'Ecologie Alpine, Université Joseph Fourier, UMR UJF-CNRS 5553, PO Box 53, F-38041 Grenoble Cedex 9, France.
E-mail: mrazpat@upjs.sk

†Present address: UMR 5202 'Origine, Structure et Evolution de la Biodiversité', Département Systématique et Evolution, Muséum National d'Histoire Naturelle, 16 Rue Buffon, F-75005 Paris, France.

‡For details see <http://www.intrabiodiv.eu>

floristic composition at the end of 19th century. It is likely that Alpine populations survived the Last Glacial in the eastern part of the Alps, from where they rapidly colonized the rest of the Alps after the ice sheet retreated. Multiple founder effects may explain a gradual loss of genetic variation during westward colonization of the Alps.

Keywords

AFLP, Alps, bottleneck, Carpathians, disjunctions, founder effect, Pleistocene glaciation, phylogeography, recolonization, vicariance.

INTRODUCTION

In Europe, alpine and subalpine plants are currently confined to several mountain ranges forming more or less separated orographic islands, which are often referred to as the 'European Alpine system' (Ozenda, 1985). Large lowland areas between the mountains constitute strong geographical barriers to gene exchange between the ranges. The disjunct distributions may be explained either by the fragmentation of a previously larger and more or less continuous distribution range or by long-distance dispersal. Quaternary climatic oscillations have greatly modified the distribution of plant species with subsequent genetic and evolutionary consequences (Comes & Kadereit, 1998, 2003; Hewitt, 2000). At the time of each climatic oscillation, with advances and retreats of continental and mountain glaciers, new habitats successively appeared and disappeared, accompanied by range shifts, fragmentations or extinctions of populations. These floristic changes have been traditionally detected from fossil remnants, and more recently from molecular data (Hewitt, 2000). During the ice ages, many temperate taxa survived in southern European glacial refugia, from where they recolonized northern regions during the interglacials (Taberlet *et al.*, 1998; Hewitt, 1999; Vogel *et al.*, 1999; Petit *et al.*, 2002). However, this paradigm cannot be applied to alpine or high mountain taxa because they have quite different ecological requirements from the species of low altitude on plains or foothills. During glacial periods, alpine taxa may have survived in non-glaciated areas in peripheral mountain refugia or in alpine nunataks (Stehlik, 2000). In the almost complete absence of macrofossils of high-mountain herbs and the difficulties of reliably assigning the pollen found in deposits to a particular species, phylogeography provides a tool for inferring the biogeographical pattern in alpine plants using molecular genetic markers (Avice, 2000). In agreement with patterns of endemism and other palaeoenvironmental data (Tribisch & Schönswetter, 2003; Tribisch, 2004), the glacial survival of several alpine plants was confirmed using molecular markers either in peripheral non-glaciated regions or possibly in central nunataks in the Alps (Schönswetter *et al.*, 2005). Refugial areas, usually representing genetically divergent zones, served as a source for rapid recolonization after glacial retreat (Schönswetter *et al.*, 2005).

The Carpathians form a large mountain arc spreading approximately from the Pannonian Gate near Bratislava

(Slovakia) to the Iron Gate in Romania. They belong to the European Alpine system and played an important role in the formation of its flora. They represent a crossroads of floristic elements from different origins (Asia, the Balkans, Central Europe) and are also characterized by a large number of endemic taxa (Pawłowski, 1970; Hendrych, 1981; Ozenda, 1995; Kliment, 1999). However, molecular studies focusing on geographical patterns of genetic structure of Carpathian populations are still lacking. High-mountain species from the Carpathian area were only sporadically included in recent phylogeographical studies, and they were usually collected in a small number of localities (usually one or two; Schneller *et al.*, 1998; Després *et al.*, 2002; Borgen & Hultgård, 2003; Kropf *et al.*, 2003; Schönswetter *et al.*, 2003, 2004a,b, 2006a,b; Alsos *et al.*, 2005; Albach *et al.*, 2006; Skrede *et al.*, 2006). It seems that Carpathian populations could serve either as a gene pool for post-glacial colonization of Scandinavia as in the case of *Trollius europaeus* (Després *et al.*, 2002), or could play the role of a 'stepping stone' in the migration of high mountain taxa from Asia to western regions of Europe (Schönswetter *et al.*, 2006a, b). Moreover, the importance of the Carpathians as a glacial refugium has also been revealed by molecular and palaeofossil studies on temperate trees (King & Ferris, 1998; Petit *et al.*, 2002; Magri *et al.*, 2006), butterflies (Schmitt & Seitz, 2001), amphibians (Babik *et al.*, 2005), snakes (Ursenbacher *et al.*, 2006) and mammals (Jaarola & Searle, 2002; Sommer & Benecke, 2004, 2005; Deffontaine *et al.*, 2005; Kotlík *et al.*, 2006).

Our main aim was to compare the genetic variation and structure of the subalpine species *Hypochaeris uniflora* in two large but disjunct parts of its European range: the Alps and the Carpathians. The species is quite common and abundant in both areas. Thus, this Alpine–Carpathian disjunction could be the result of long-term isolation of populations in both ranges. In such a case, a high level of genetic differentiation between both regions would be expected. However, the populations of *H. uniflora* would have experienced different glacial histories in the two mountain ranges, which might have had an impact on the overall genetic variation and structure. While the Alps were repeatedly covered by a massive continuous ice sheet, only local glaciers occurred in the highest part of the Carpathians, usually above 2000 m (Charlesworth, 1957), and most of the Carpathians remained free of ice even during the coldest periods. Thus, high-mountain plants of the Carpathians might

have experienced lower fragmentation of suitable habitats and depauperation of genetic diversity than in the Alps. Nonetheless, we may expect more frequent migration of the high-mountain flora between the Alps (or the nearby refugia where the Alpine flora survived) and the Carpathians during the long glacial periods than during the short warm interglacial periods. Indeed, because of the extremely cold and dry climate, the timberline was much lower than today and the continuous forest biomes, which form large natural barriers to the dispersal of high-mountain plants, were largely absent from Central Europe (Ray & Adams, 2001). In the Alps, the existence of several glacial refugia has been reported for many high-mountain taxa (Schönswetter *et al.*, 2005). Is this also the case for the subalpine *H. uniflora*? To infer the phylogeographical history of the species in the Alps and the Carpathians, we used a large number of amplified fragment length polymorphism (AFLP; Vos *et al.*, 1995) loci as a tool for estimating the genetic similarity between individuals and populations.

MATERIAL AND METHODS

Species

Hypochaeris uniflora Vill. (*Hypochaeris* section *Achyrophorus* Scop., Asteraceae) is a 20–50 cm tall, perennial herb with rosette leaves. It has one yellow capitulum of 25–50 mm in diameter and is insect pollinated. Long-distance seed dispersal may be possible by means of the beaked achenes with pappus (Sheldon & Burrows, 1973; Andersen, 1993). *Hypochaeris uniflora* is confined to the subalpine belt in the Alps,

Carpathians and Sudetes Mountains (Fig. 1), where it grows on primary or secondary grasslands from (700–)1400 to 2600 m a.s.l., and usually on siliceous bedrock (De Filippis, 1976; Meusel & Jäger, 1992). It is characteristic of plant communities dominated by *Nardus stricta* and *Calamagrostis villosa* (Kliment *et al.*, 2007). Only diploids ($2n = 2x = 10$) are known (Májovský *et al.*, 1987; Dobeš & Vitek, 2000).

Sampling

Hypochaeris uniflora was sampled across the Alps and Carpathians, covering almost the whole distribution range of the species. The sampling strategy followed the protocol adopted for the IntraBioDiv project, with leaf samples collected in 25 × 25 km regular grid squares following Niklfeld (1971) (Table 1, Fig. 2a). The leaves of three individual plants per population were collected at a distance of at least 8–10 m apart, dried in silica gel and stored at room temperature. In 33 populations, one random plant was collected twice to test the reproducibility of AFLP analyses (Bonin *et al.*, 2004). Voucher specimens from some populations were deposited in the herbarium of the Institut für Systematische Botanik in Zürich (Z).

DNA extraction and AFLP analyses

Total DNA was extracted from 10–15 mg of silica-dried leaf tissue with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Double digestion of genomic DNA was performed for 2 h at 37°C in a 20 µl mix using 2 U of *Mse*I and 5 U of *Eco*RI (New England Biolabs,

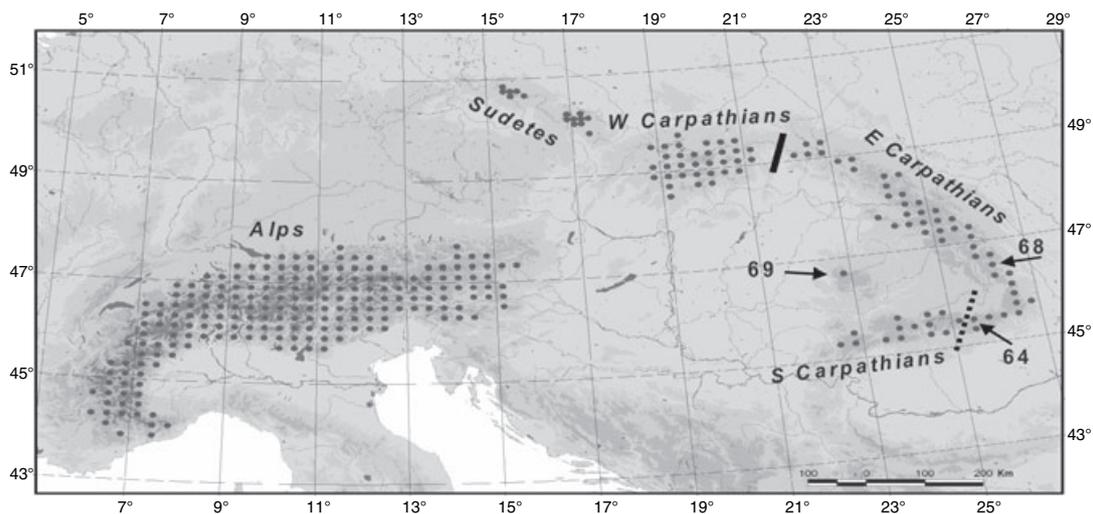


Figure 1 Distribution range of *Hypochaeris uniflora* Vill. The distribution in the Alps and the Carpathians is based on mapping performed as part of the IntraBioDiv project. Dots correspond to 25 × 25 km squares. The distribution in the Sudetes Mountains was plotted with a finer grid scale according to a recent map published by Slavík (2004). The full line represents the phytogeographical boundary between the eastern and western Carpathians, first recognized by Wołoszczak (1896), whereas the dashed line represents the geomorphological boundary between the eastern and southern Carpathians. Arrows with numbers show the location of populations sampled in the eastern Carpathians (no. 68, Nemira Mountains; no. 64, Ciucuş) but belonging to the southern Carpathians phytogeographical group according to the AFLP data, and an isolated population from the Apuseni Mountains (no. 69).

Table 1 Geographical origin of the 77 investigated populations of *Hypochaeris uniflora* in the Alps and Carpathians and within-population genetic variation expressed as the number of amplified fragment length polymorphism (AFLP) bands in population (Nbds), percentage of polymorphic bands (%Poly) and mean pair-wise Jaccard distance (JC). Frequency-down-weighted fragment values (DW) are given for the whole data set (DW_{total}), and separately for Alpine populations only (DW_{Alps} , see Material and Methods). Each population was represented by three plants.

Code	Country	Locality	Collector(s)	Long.	Lat.	Nbds	%Poly	JC	DW_{total}	DW_{Alps}
1	I	Colle di Sampeyre	SP	7.12	44.55	45	33.3	0.480	0.97	1.50
2	I	Colle Vaccera	SP	7.20	44.87	45	17.8	0.347	1.02	1.56
3	I	Bassa Valle di Susa, pascoli presso il Colle del Collombardo	CM	7.31	45.19	47	27.7	0.434	1.35	2.20
4	I	Costa Pianard – Vernante	PF	7.47	44.20	43	34.9	0.485	0.77	1.01
5	I	Valle Soana, pascoli sotto Grange Arietta	AT	7.50	45.57	48	25.0	0.415	1.22	1.51
6	CH	Alpes Pennines, Chandolin, Arête des Ombrintses	PD, PK, GM	7.61	46.25	45	35.6	0.497	0.72	1.10
7	CH	Alpes Pennines, Simplon Pass	PD, PK, GM	8.03	46.25	42	16.7	0.332	0.67	0.88
8	I	Alpes Lépointiennes, Alpe Capezzone, Verbania, Vallestrona	RDV	8.22	45.93	49	32.7	0.475	0.80	1.23
9	I	Sankt Gothard Pass, southern slopes	PD, PK, GM	8.56	46.53	43	25.6	0.428	0.63	0.81
10	CH	Oberal Pass	PD, PK, GM	8.68	46.66	43	25.6	0.417	0.66	0.88
11	CH	Erbserstock	PD, PK, GM	9.09	46.90	44	27.3	0.434	0.67	0.92
12	I	Lariani Mountains, Valle di San Iorio	GP	9.16	46.16	41	26.8	0.430	0.62	0.83
13	I	Pressi Zuc di Valbona	GP	9.49	46.01	48	37.5	0.516	1.39	2.42
14	CH	Graubünden, Parpaner Weisshorn	PTa	9.59	46.76	50	30.0	0.460	0.86	1.42
15	L	Malbun, Fürstentum	FG	9.61	47.08	47	36.2	0.499	0.74	0.98
16	I	Mount Monte Rolla	GP	9.83	46.20	44	36.4	0.509	0.61	0.79
17	CH	Graubünden, Marjuns	PTa	9.83	46.52	47	34.0	0.485	0.87	1.55
18	CH	Graubünden, Dörfli	PTa	9.89	46.81	49	30.6	0.460	1.33	1.66
19	CH	Graubünden, Val Flesse	PTa	10.01	46.77	46	32.6	0.470	0.77	1.24
20	I	Piancamuno, Montecampione 2	IB	10.24	45.84	47	27.7	0.435	0.76	1.07
21	A	Sankt Anton	CT	10.29	47.16	46	30.4	0.458	0.70	0.91
22	I	Monno, pascolo a Nord del Passo Mortirolo	IB	10.30	46.25	50	34.0	0.484	0.83	1.27
23	I	Collio	EB	10.34	45.79	47	29.8	0.450	0.90	1.93
24	A	Allgäuer Alpen, Lechtal	FG	10.37	47.27	46	26.1	0.423	0.80	1.10
25	I	Ressia, Mount Piz Lat	PTa	10.48	46.84	46	28.3	0.442	0.76	1.24
26	I	Ortler, Alpen, Santa Caterina, Refugia del Forni	PTa	10.56	46.42	47	25.5	0.421	0.80	1.27
27	I	Ötztaler Alpen, below Gieshof	PTa	10.67	46.72	47	31.9	0.464	0.74	1.08
28	I	Tiarno di Sotto	AB	10.68	45.93	47	36.2	0.500	0.72	0.96
29	I	Gruppo della Presanella: Val d'Amola	AB, AA	10.71	46.21	47	27.7	0.436	0.87	1.28
30	A	Ötztaler Alpen, Trenkroald	PTa	10.87	47.01	46	30.4	0.461	0.72	0.95
31	I	Lessini Mountains, W of the village of Vo Sinistro	AB	11.06	45.69	43	20.9	0.379	0.65	0.88
32	I	Gruppo delle Maddalene: Mount Cornicolo	AB	11.07	46.50	47	29.8	0.451	0.81	1.12
33	I	Timmelsjoch road	PTa	11.12	46.89	49	28.6	0.448	0.85	1.44
34	I	Prealpi Trentine, Mount Panarotta	AB, FP	11.33	46.05	48	31.2	0.464	0.82	1.32
35	I	Stubai Alpen, Riobianco	AH	11.38	46.78	49	30.6	0.459	0.80	1.13
36	I	Novaledo, SE of the village	SS	11.38	45.98	49	28.6	0.440	1.79	2.09
37	A	Valsertal	CT	11.50	47.04	49	26.5	0.419	1.10	1.69
38	I	Dolomits, Stava	FP	11.55	46.35	47	34.0	0.482	0.70	0.95
39	I	Dolomits, Val Gardena, S of the village of Selva	PMa	11.76	46.51	47	34.0	0.483	0.74	1.01
40	I	Dolomits, W of the village of Mount Croce	CL	11.80	46.09	51	43.1	0.543	0.98	1.98
41	A	Zillertal, Tuxer Voralpen	CT	11.84	47.24	49	30.6	0.460	0.78	1.08
42	I	Ahrntal	KS	12.13	47.06	54	33.3	0.483	1.39	2.02
43	I	Planca di Sotto	PMa	12.23	46.77	46	23.9	0.403	0.78	1.04
44	I	Dolomits, Ciabana	CA	12.25	46.38	48	39.6	0.528	0.88	1.62
45	I	Dolomits, Cima Gogna	CA	12.41	46.51	46	26.1	0.415	0.77	1.05
46	A	Hohe Tauern, Mattersberg, SE of the village	MB	12.58	46.99	45	35.6	0.489	0.63	0.85
47	A	Hohe Tauern, Winkl Heiligenblut, N	CT	12.83	47.06	46	15.2	0.314	1.74	2.33
48	A	Hohe Tauern, Häusler Alm – Lonzaköpfl	BF	13.15	46.98	49	32.7	0.474	0.88	1.36
49	A	Schladminger Tauern, Preber S Roßscharte	SE	13.87	47.21	49	32.7	0.473	1.11	1.52
50	A	Gurktaler Alpen, Turracherhöhe	BF	13.88	46.92	51	49.0	0.584	1.00	1.71

Table 1 continued.

Code	Country	Locality	Collector(s)	Long.	Lat.	Nbds	%Poly	JC	DW _{total}	DW _{Alps}
51	A	Steiermark, Frauenalpe, SW of the summit	SE	14.14	47.06	52	30.8	0.466	1.29	1.93
52	A	Wölzer Tauern, Klosterneuburger Hütte	SE	14.38	47.27	49	32.7	0.465	0.95	1.95
53	A	Stubalpe, Speikkogel	TE, MVL	14.85	47.05	52	36.5	0.501	1.34	2.09
54	A	Koralpe, Godinger Wald – Gr. Speikkogel	TB, NJ	14.95	46.79	50	32.0	0.475	1.26	1.91
55	A	Gleinalpe, Mount Speikkogel	MVL, TE	15.05	47.22	48	31.2	0.455	0.89	1.37
56	S	Krivánska Malá Fatra Mountains, Mount Veľký Fatranský Kriváň – Snilovské sedlo	RL, MPe	19.03	49.19	46	30.4	0.463	1.20	
57	S	Oravské Beskydy Mountains, Mount Babia Hora	RL, PMr	19.52	49.57	45	42.2	0.542	1.19	
58	S	Nízke Tatry Mountains, Mount Ďumbier	RL	19.65	48.93	53	45.3	0.576	1.63	
59	S	Západné Tatry Mountains, Banikovské Sedlo	RL, PMr	19.72	49.20	49	46.9	0.573	1.49	
60	S	Nízke Tatry Mountains, Mount Kráľova Hoľa	RL, PTu, PMr	20.15	48.88	46	41.3	0.541	1.17	
61	S	Vysoké Tatry Mountains, Tatranská Polianka – Sliezky Dom	MR, PS	20.17	49.14	44	40.9	0.536	0.97	
62	R	Retezat Mountains, Mount Buta	ZS	22.90	45.33	44	34.1	0.484	2.32	
63	R	Bucegi Mountains, Valea Jepilor	MR, AR	25.49	45.41	41	39	0.518	1.45	
64	R	Ciucă Mountains, Culmea Gropșoarele	GC, MPu	25.98	45.49	56	57.1	0.657	2.12	
65	R	Șureanu Mountains, Mount Șureanu	EC, JC, LW	23.50	45.58	44	43.2	0.553	1.52	
66	R	Cindrel Mountains, Iezerul Mare	EC, JC, LW	23.80	45.59	42	31.0	0.465	1.71	
67	R	Făgăraș Mountains, Mount Pietra Caprei	MR, AR	24.80	45.63	44	27.3	0.428	2.11	
68	R	Nemira Mountains, Mount Nemira Mare	GC, MM	26.32	46.26	51	47.1	0.583	2.53	
69	R	Apuseni Mountains, Mount Cârlița	VC, GC, MPu	22.67	46.64	45	60.0	0.664	1.90	
70	R	Ceahlău Mountains, under Mount Toaca	IS, AO	25.95	46.98	46	52.2	0.614	1.45	
71	R	Călimani Mountains, Mount Pietricelul	IS, AO	25.23	47.10	45	42.2	0.541	1.33	
72	R	Giumalău Mountains, Mount Giumalău	IS, AO	25.48	47.43	47	44.7	0.564	1.37	
73	R	Rodna Mountains, Mount Corongiș – Mount Saca	GC, MPu	24.80	47.51	48	54.2	0.622	2.52	
74	R	Maramureș Mountains, Mount Torioioaga	RL, MPe	24.73	47.73	53	58.5	0.656	2.67	
75	U	Chornohora Mountains, Mount Pozhzhzhevskya	RL, NS	24.54	48.16	43	72.1	0.743	1.11	
76	U	Gorgany Mountains, Mount Berezovacky Klyvky	RL, NS	24.32	48.46	47	57.4	0.643	1.10	
77	U	Polonina Borzhava Mountains, Mount Velikiy Verkh	RL, NS	23.33	48.65	48	39.6	0.525	1.94	

Country abbreviations: A, Austria; CH, Switzerland; I, Italy; L, Liechtenstein; R, Romania; S, Slovakia; U, Ukraine.

Collectors abbreviations: AA, A. Angheben; AB, A. Bertolli; AH, A. Hilpold; AO, A. Oprea; AR, A. Ronikier; AT, A. Tisi; BF, B. Frajman; CA, C. Argenti; CL, C. Lasen; CM, C. Minuzzo; CT, C. Thiel; EB, E. Bonna; EC, E. Cieślak; FG, F. Gugerli; FP, F. Prosser; GC, G. Coldea; GM, G. Mansion; GP, G. Parolo; IB, I. Bonna; IS, I. Sârbu; JC, J. Cieślak; KS, K. Steinmann; LW, L. Wilk; MB, M. Bucceri; MM, M. Miclăuș; MPe, M. Perný; MPu, M. Pușcaș; MR, M. Ronikier; MVL, M. van Loo; NJ, N. Jogan; NS, N. Sychak; PD, P. Druard; PF, P. Fantini; PK, P. Kűpfer; PMA, P. Mair; PMr, P. Mráz; PS, P. Schönewetter; PTA, P. Taberlet; PTu, P. Turis; RDV, R. della Vedova; RL, R. Letz; SE, S. Ertl; SP, S. Picco; SS, S. Scortegagna; TB, T. Bačić; TE, T. English; VC, V. Cristea; ZS, Z. Szeląg.

Ipswich, UK). Following this, adaptors were ligated to DNA in a 40 μ l volume for 2 h at 37°C using 1 U of T4 DNA Ligase (New England Biolabs). After a dilution (1 : 10) of digested-ligated products, DNA was submitted to a pre-selective polymerase chain reaction (PCR) amplification using primers EA (5'-GACTGCGTACCAATTCA-3') and MC (5'-GACTGCGTACCAATTCA-3'), in a 25 μ l volume containing 1.5 mM MgCl₂, 200 μ M of each nucleotide (dNTP), 1.25 μ M of each primer and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) with the following cycle profile: 72°C/2 min; 94°C/30 s, 56°C/30 s, 72°C/2 min (30 cycles); 72°C/10 min and 4°C thereafter. After a 1 : 20 dilution of pre-selective PCR products, DNA underwent selective PCR with the following primer combinations: *EcoRI*-ACA/*MseI*-CAC; *EcoRI*-AGG/*MseI*-CTG; *EcoRI*-ACC/*MseI*-CAG. Selective amplifications

were run in a 25 μ l volume containing 2.5 mM MgCl₂, 200 μ M of each dNTP, 1.25 μ M of each primer and 1 U of AmpliTaq Gold[®] DNA polymerase (Applied Biosystems). Amplifications were performed with the following profile: 95°C/10 min; 94°C/30 s, 65 \rightarrow 55.9°C ($\Delta T = -0.7^\circ\text{C}/\text{cycle}$), 72°C/1 min (13 cycles); 94°C/30 s, 56°C/1 min, 72°C/1 min (23 cycles); 72°C/10 min and 4°C thereafter. After a spin-column purification, 1 μ l of the diluted (1 : 10) purified product was mixed with 10 μ l of HiDi formamide (Applied Biosystems) and electrophoresed for 40 min on an ABI PRISM[®] 3100 Genetic Analyzer (Applied Biosystems) using 36 cm capillaries and POP-4[®] polymer. Before importing the data in GENOGRAPHER, fragment analyses were size called in GeneScan version 3.7. (Applied Biosystems). Fragments of size range 50–500 base pairs (bp) were manually scored with GENOGRAPHER version 1.6 (Montana State Univer-

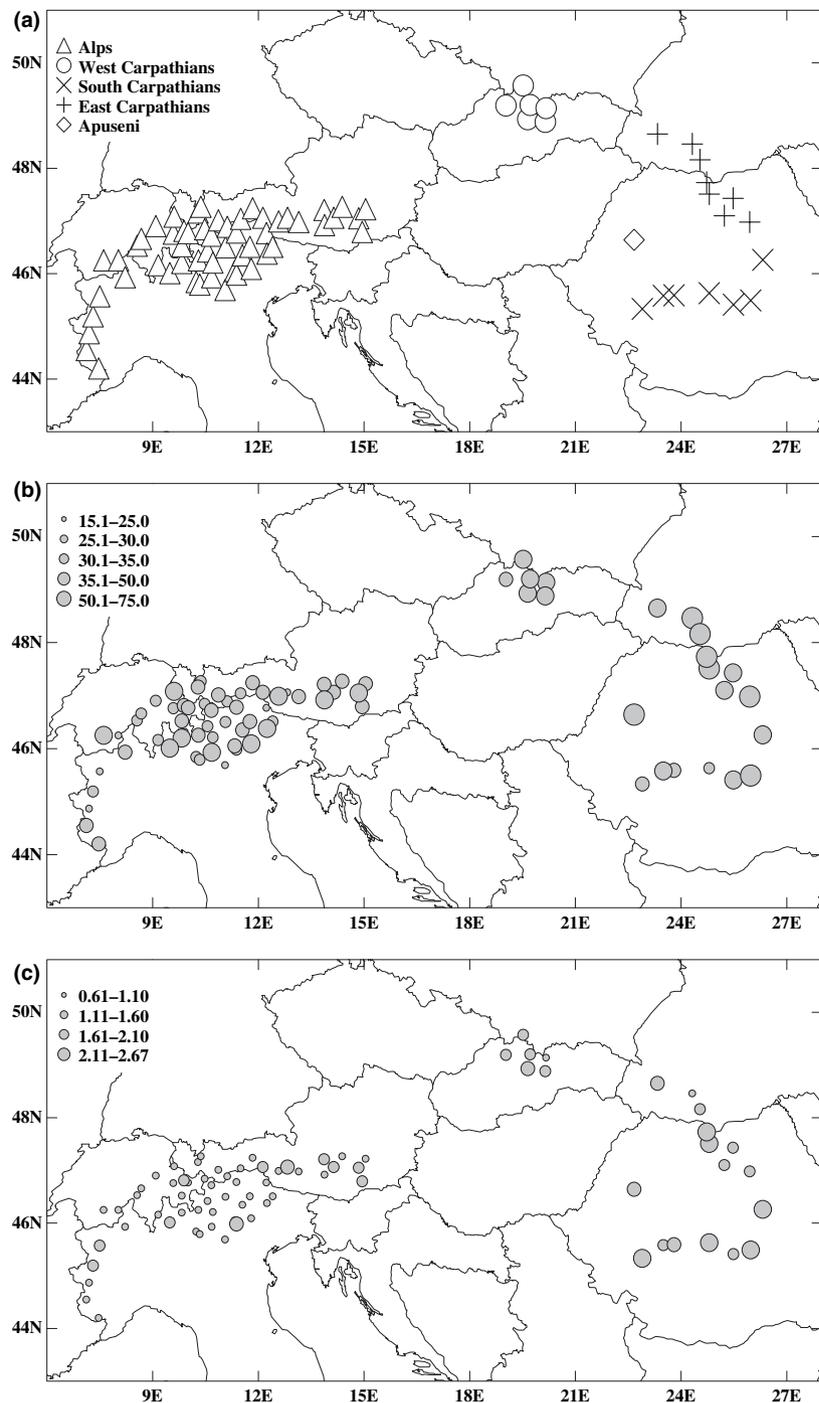


Figure 2 (a) Geographical origin of the 77 analysed populations of *Hypochaeris uniflora* covering the whole distribution range of the species, with the exception of small isolated areas in the Sudetes Mountains. Different symbols represent different groups detected by principal coordinate and neighbour joining analyses based on 87 amplified fragment length polymorphism (AFLP) markers. (b) Level of within-population variation expressed as the percentage of polymorphic loci (%). (c) 'Frequency-down-weighted fragment values' per population (DW) used for the estimation of the proportion of rare AFLP fragments within populations. Maps were created using DMAP (Morton, 2004).

sity 1998; <http://hordeum.oscs.montana.edu/genographer>) for their presence/absence.

Data analysis

All monomorphic bands and bands that were observed in only one or in all but one plant were removed from the data set to avoid the inclusion of possibly artefactual bands. We calculated the total number of AFLP fragments, the percentage of

polymorphic fragments, and the mean pair-wise Jaccard distance among individuals to characterize within-population variation (software R-cran; R Development Core Team, 2006). We found only two populations with population-specific fragments (a population-specific fragment is present in only one population, but not necessarily in all its samples), probably due to the low sampling size within each population and very strict choice of polymorphic bands (see above). In order to take into account the overall frequency of fragments when

assessing their rarity within each population, we computed 'frequency-down-weighted fragment values' per population (DW), which is equivalent to range-down-weighted species values or 'weighted endemism' in biodiversity studies (Crisp *et al.*, 2001; Schönswetter & Tribsch, 2005). Within each population, and for each fragment, the DW parameter was computed as the frequency of a given AFLP fragment in the population divided by the overall frequency of the marker in the whole data set. Thus, DW values are high for rare fragments and substantially lower for bands that are common in the overall data set. DW parameters were summed over all fragments to provide a population-level index. High values of DW are expected in long-term isolated populations, whereas recently diverged populations should exhibit low DW values due to the founder effect (Schönswetter & Tribsch, 2005).

To infer the phylogeographical history of Alpine populations in more detail, we also computed DW values for Alpine populations only, using a subset of the data only including Alpine populations (and including AFLP fragments present only in Alpine populations), as the high number of rare fragments (high DW values) found in the Carpathian populations might partially bias DW values in the Alps. Moreover, we tested the possible correlations between geographical location of populations in the Alps and within-population genetic variation. Pearson correlation coefficients were computed using a basic statistics package implemented in R-cran (R Development Core Team, 2006).

A principal coordinate analysis (PCoA) based on inter-individual Jaccard distances was computed using SYN-TAX 2000 (Podani, 2001). At the population level, an unrooted neighbour-joining (NJ) tree was constructed with PHYLIP version 3.6 (Felsenstein, 2005) based on pair-wise population genetic distances (according to Lynch & Milligan, 1994) computed with AFLPSURV (Vekemans, 2002) and visualized by TreeView (Page, 1996). Considering AFLP markers as biallelic (presence/absence), allelic frequencies were estimated using two methods: the square-root method and the Bayesian method with non-prior distribution, both implemented in AFLPSURV. The robustness of the tree was tested by 1000 bootstrap replicates over the loci. Analyses of molecular variance (AMOVA) based on the pair-wise unmodified Nei and Li distance (Nei & Li, 1979) within and between populations were carried out in ARLEQUIN version 3.0 (Excoffier *et al.*, 1992) to test the partitioning of genetic variation within and between the main groups identified by NJ and PCoA.

We performed one-way analyses of variance (ANOVA) to test for a geographical effect on within-population diversity indices. According to the results from NJ, PCoA and AMOVA, populations were pooled into three (Alps, western and south-eastern Carpathians) or four (southern and eastern Carpathians treated separately) main geographical regions. To account for a possible bias due to the unbalanced sampling effort among regions, we used re-sampling procedures to generate a large number of data sets comprising six populations per region (this number was limited by the lowest number of

populations analysed within a region, namely in the western Carpathians). Furthermore, we examined whether the frequency of each polymorphic marker was independent of the geographical region. This analysis was based on presence/absence data at the population level, i.e. a given AFLP fragment was considered present in a population if one individual at least exhibited that marker. For each contingency table (regions \times presence/absence data), we generated an exact distribution of the χ^2 statistic using randomization tests (1000 replicates), and compared the observed value with this exact distribution. The procedure was implemented in the software R-cran (R Development Core Team, 2006). In the case of lack of independence in contingency tables, we computed the standardized residual of each cell using the formula: $(\text{Obs} - \text{Exp})/(\text{Exp})^{1/2}$ where 'Obs' and 'Exp' are observed and expected frequencies respectively. A threshold of 1.96 was chosen to indicate a significant deviation from independence, i.e. a higher frequency of the fragments than expected for a given region. We then observed whether these region-diagnostic fragments were found in one region only or, alternatively, shared by several regions.

RESULTS

We scored 87 polymorphic bands in 77 populations of *H. uniflora*. Repeatability was very high (98%).

The PCoA revealed the existence of three well-separated groups of plants representing three geographical regions: the Alps and the western and south-eastern Carpathians (Fig. 3). In addition, the plants from the southern Carpathians could be distinguished from those from the eastern Carpathians, whereas the plants from the geographically intermediate Apuseni Mountains (population 69) were intermingled with these two subgroups (Fig. 3). The genetic split between the southern and eastern Carpathians roughly follows the phyto-geographical division of the Romanian Carpathians (Georgescu & Doniță, 1965; Beldie, 1967). However, the plants from the Ciucaș and Nemira ranges (populations 64 and 68 respectively; Figs 1 & 2a) were placed in the southern Carpathian group based on AFLP data, whereas they belong to the eastern Carpathians based on phytogeography and geomorphology.

The same pattern of genetic differentiation was obtained by the NJ analysis based on genetic distances among populations (Fig. 4). Three clearly separated groups had high bootstrap support. The subdivision of the south-eastern Carpathian branch into eastern and southern subgroups was moderately supported, with a more or less intermediate position of the population from the Apuseni Mountains (Fig. 4). However, when we omitted the Apuseni population from the NJ analysis, the eastern-southern Carpathians division had high bootstrap support (94%, data not presented). Two different approaches used for computation of allelic frequencies (root-square method and Bayesian method) provided the same NJ tree topology with similar bootstrap support for the main groups (Fig. 4). However, the Bayesian approach showed bootstrap values above 50% for two additional pairs of populations: one

Figure 3 Principal coordinate analysis (PCoA) plot of 231 individuals from 77 populations of *Hypochaeris uniflora* based on Jaccard distances calculated on amplified fragment length polymorphism (AFLP) multilocus phenotypes. A total of 24.0% of the total genetic variation was explained by the first two axes.

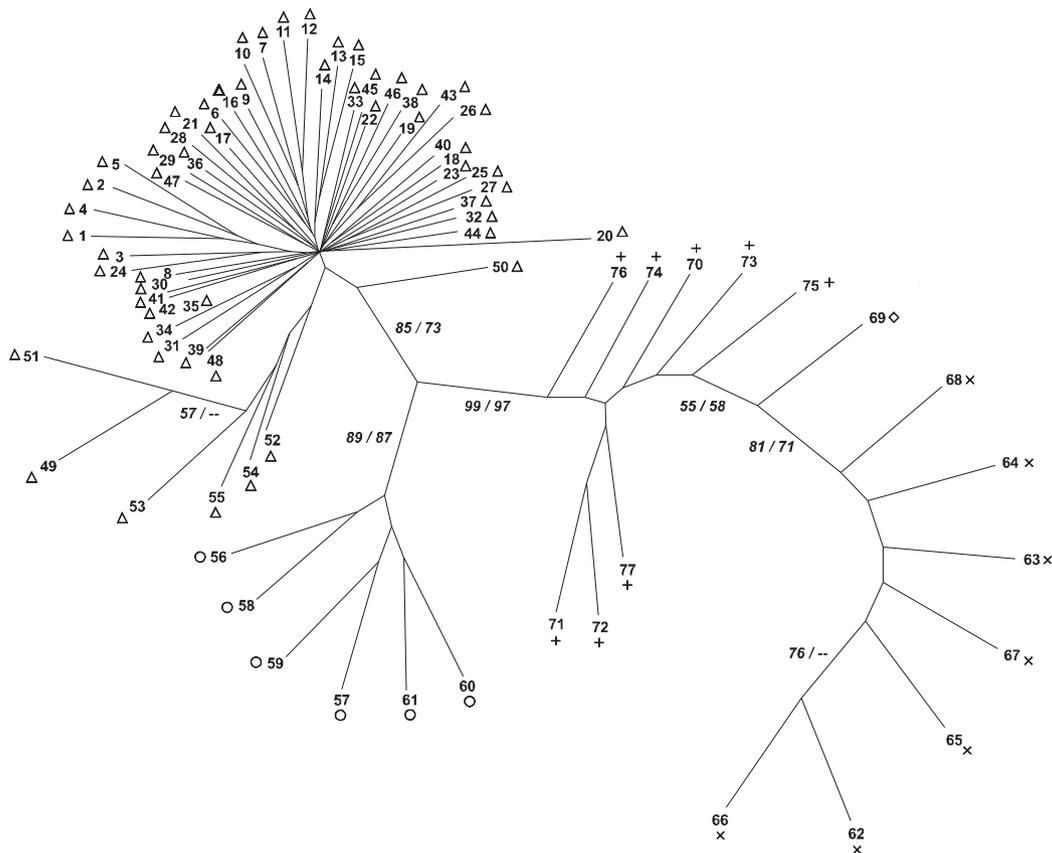
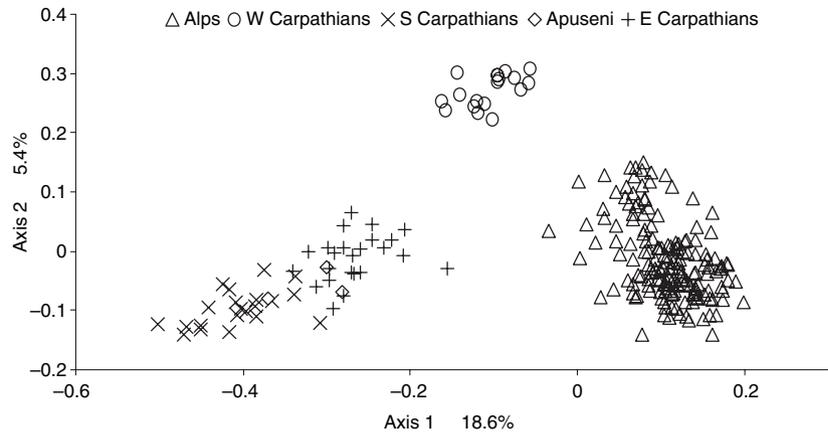


Figure 4 Neighbour joining unrooted tree based on genetic distance among 77 populations of *Hypochaeris uniflora*. The numbers at the tips of branches are population numbers (see Table 1) and the symbols are the same as in Figs 2 & 3: Δ , Alps; \circ , western Carpathians; \times , southern Carpathians; $+$, eastern Carpathians; \diamond , Apuseni Mountains. The numbers above the branches are bootstrap values higher than 50% (based on 1000 replicates). The two bootstrap values correspond to the computations based on the matrix of genetic distances computed with and without a Bayesian approach. The major topology of the tree was the same in both cases.

pair of populations from the eastern Alps and one pair of populations from the southern Carpathians.

In the AMOVA without grouping populations, 42.5% of the total genetic variation was found between populations (Table 2). When assuming four groups of populations

(Alps, western, southern and eastern Carpathians), 45.9% of the variation was observed between groups of populations and 10.6% was found among populations within groups (Table 2). These percentages of variation were 43.2% and 13%, respectively, when assuming three groups (merging the

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
All populations	Among populations	76	1347.91	4.07	42.45
	Within populations	154	850.00	5.52	57.55
Three groups: Alps, W Carpathians, SE Carpathians	Among groups	2	575.62	5.45	43.23
	Among populations	74	772.29	1.64	13.00
Four groups: Alps, W, S, E Carpathians	Within populations	154	850.00	5.52	43.77
	Among groups	3	650.06	5.82	45.88
	Among populations	73	697.85	1.35	10.61
Alps	Within populations	154	850.00	5.52	43.51
	Among populations	54	476.06	1.32	21.48
W Carpathians	Within populations	110	532.67	4.84	78.52
	Among populations	5	43.50	0.73	10.14
SE Carpathians	Within populations	12	78.00	6.50	89.86
	Among populations	15	252.73	3.12	29.46
S Carpathians	Within populations	32	239.33	7.48	70.54
	Among populations	7	84.71	1.83	21.60
E Carpathians	Within populations	16	106.00	6.63	78.40
	Among populations	7	93.58	1.68	16.77
	Within populations	16	133.33	8.33	83.23

Table 2 Analyses of molecular variance (AMOVA) of the 77 investigated populations of *Hypochaeris uniflora* in the Alps and Carpathians. When the southern and eastern Carpathian groups were treated separately, the population from the Apuseni Mountains was included in the southern Carpathian group.

Table 3 Comparison of within-population diversity indices among different phylogeographical groups delineated by neighbour joining, principal coordinate and analysis of molecular variance (AMOVA) analyses in *Hypochaeris uniflora*. After the resampling procedure, each particular group comprised six populations (see Material and Methods). Means and standard errors with the same letters are not significantly different (ANOVA, Tukey pair-wise comparison, all $P < 0.001$).

Group	Npop	Nbds _{tot}	Nbds/pop ₆	%Poly/pop ₆	JC/pop ₆	DW _{total} /pop ₆
Three-groups model						
Alps	55	73	47.01 ± 0.95 a	30.46 ± 2.42 a	0.458 ± 0.020 a	4.15 ± 0.22 a
W Carpathians	6	63	47.17 ± 0.00 a	41.17 ± 0.00 b	0.539 ± 0.000 b	4.02 ± 0.10 b
SE Carpathians	16	77	46.41 ± 1.33 b	47.24 ± 4.12 c	0.581 ± 0.030 c	5.38 ± 0.24 c
Four-groups model						
Alps	55	73	47.16 ± 1.06 a	30.50 ± 2.55 a	0.459 ± 0.020 a	3.50 ± 0.23 a
W Carpathians	6	63	47.17 ± 0.00 a	41.17 ± 0.00 b	0.539 ± 0.000 b	3.16 ± 0.07 b
S Carpathians	8	72	45.77 ± 1.03 b	41.90 ± 1.80 b	0.544 ± 0.017 b	3.62 ± 0.11 c
E Carpathians	8	73	47.14 ± 0.59 a	52.98 ± 2.16 c	0.615 ± 0.013 c	3.43 ± 0.15 a

Npop, number of analysed populations in each particular group; Nbds_{tot}, total number of amplified fragment length polymorphism (AFLP) bands per group; Nbds/pop₆, average number of AFLP bands per population after resampling; %Poly/pop₆, average percentage of polymorphic AFLP fragments per population after resampling; JC/pop₆, main pair-wise Jaccard distance between individuals within population after resampling; DW_{total}/pop₆, average proportion of frequency-down-weighted fragment values (counted from the whole data set) per population after resampling.

southern and eastern Carpathians; Table 2). Separate AMOVAs performed within each geographical region revealed the highest among-population variation in the south-eastern Carpathian group (29.5%; Table 2) and the lowest among-population variation in the western Carpathians (10.1%; Table 2).

The highest within-population genetic diversity was found in the population from Mount Pozhzyzhevska (no. 75, eastern Carpathians), whereas the lowest genetic diversity was found in the population from the Simplon Pass (no. 7, Central Alps; see also Fig. 2b). Carpathian populations usually exhibited higher proportions of rare AFLP bands (higher DW values) than Alpine populations (Fig. 2c, Table 1). Average within-population genetic variation in all parts of the Carpathians

was significantly higher than in the Alps, and the largest values were found in the eastern Carpathians (Table 3). Moreover, the group of Alpine populations had fewer region-diagnostic markers than groups of Carpathian populations (18, 4 and 3 region-diagnostic markers for the south-eastern Carpathians, western Carpathians and Alpine groups respectively; Table 4), despite the larger geographical extent of the Alpine group. While seven region-diagnostic markers were shared by southern and eastern Carpathian populations, showing their close genetic relationships, only one region-diagnostic marker was common for the whole Carpathians. No region-diagnostic marker was shared between the Alps and any of the Carpathian regions (Table 4). Interestingly,

Table 4 Number of region-diagnostic amplified fragment length polymorphism (AFLP) fragments of *Hypochoeris uniflora* in different phylogeographical regions (out of a total of 87 markers). The numbers of region-diagnostic AFLP fragments shared by several regions are given in brackets.

Geographical region	Three-regions model	Four-regions model
Alps	3	3
Western Carpathians	4 + (1 WSE)	4 + (1 WSE)
South-eastern Carpathians	18 + (1 WSE)	–
Southern Carpathians	–	6 + (1 WSE) + (7 SE)
Eastern Carpathians	–	6 + (1 WSE) + (7 SE)

WSE denotes region-diagnostic fragments shared by the western, southern and eastern Carpathians. SE denotes region-diagnostic fragments shared by the southern and eastern Carpathians.

while the populations from the eastern Carpathians were more variable than those from the southern Carpathians (significantly higher percentage of polymorphic loci and mean pair-wise Jaccard distance within population), the latter harboured higher proportions of rare AFLP fragments (higher DW values; Table 3, Fig. 2b,c).

No clear phylogeographical structure was found in the Alps. Although the PCoA suggested some grouping of individual plants from easternmost populations, this structure had low (< 50%) bootstrap support on the NJ tree constructed at the population level (except for two populations; Fig. 3). In the NJ tree, the Alpine populations form a star-like homogeneous cluster. Despite almost no phylogeographical pattern in the Alps, we found a strong and significant correlation ($r = 0.568$, $P < 0.001$) (Fig. 5) between the total number of AFLP bands per population and the longitude, with the highest values in the eastern Alps and the lowest values in the western Alps (Fig. 4). The other within-population diversity parameters, e.g. DW values, proportion of polymorphic loci and mean pair-wise Jaccard distance, were also correlated in the same manner ($r = 0.302$, 0.271 and 0.245 respectively; all $P < 0.05$). Besides

the eastern Alps, the highest DW values within Alpine populations were found along the southern border of the range (Fig. 2c).

DISCUSSION

Our AFLP data revealed a strong phylogeographical pattern within the subalpine species *H. uniflora*. There was a clear genetic split between three groups of populations belonging to three different geographical regions: the Alps, western Carpathians and south-eastern Carpathians. Although *H. uniflora* has long-distance dispersal mechanisms (i.e. achenes with hairy pappus), the patterns of AFLP variation within the species indicate the absence of recent long-distance dispersal since all individuals and populations were clustered according to their geographical origin. This may suggest long-term isolation of populations and the evolution of distinct genetic lineages with substantial accumulation of differences in the genome (Avice, 1987).

Differentiation among Carpathian populations

Based on our AFLP data, the western Carpathian populations of *H. uniflora* were clearly separated from other Carpathian populations. Although the Carpathians form a continuous mountain range, an important phylogeographical line roughly follows the geomorphological boundary between the western and eastern Carpathians. First recognized by Wołoszczak (1896), the transition area of the Skhidni Beskidi Mountains (the westernmost part of the eastern Carpathians slightly extending into Poland, here called Bieszczady Mountains, and Slovakia, here called Bukovské vrchy Mountains) has represented a strong barrier to the dispersal of many montane and (sub)alpine plant species from the eastern to the western Carpathians and vice versa (Fig. 1). A peculiarly warm climate and the low altitude of the main ridge of the Skhidni Beskidi Mountains (the lowest parts are c. 700 m a.s.l. only) were probably the main causes of this disjunction (Domin,

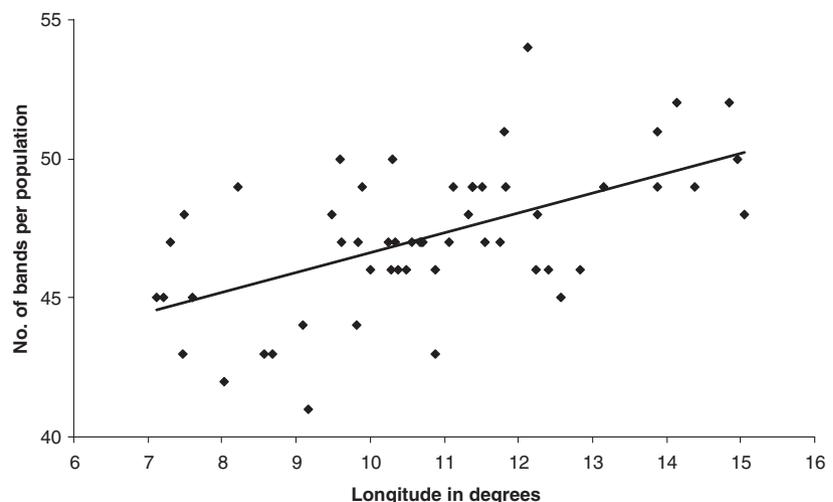


Figure 5 Correlation between the total number of amplified fragment length polymorphism (AFLP) bands per population of *Hypochoeris uniflora* and the longitude of sampled sites in the Alps ($n = 55$, $r = 0.568$, $P < 0.001$; two-tailed probabilities).

1940–1941). The westernmost localities of *H. uniflora* in the eastern Carpathians are also situated in this mountain range (although not analysed in the present study, the geographically close population 77-Polonina Borzhava was included in AFLP analyses) and are separated from the closest western Carpathian populations by c. 90 km (Fig. 1). Thus, in agreement with floristic (Pax, 1898; Jasiewicz, 1965; Malinovskii, 1991) and cytogeographical evidence (Mráz & Szeląg, 2004), our results show the existence of a strong genetic barrier between the western and eastern Carpathians.

It is not possible to date this separation using AFLP markers as the rate of mutation is unknown. On the one hand, some palaeovegetation studies suggested the existence of a continuous alpine–subalpine bridge between the western and eastern Carpathians during the late phases of the last glacial period (Würm; Krippel, 1986; Zagwijn, 1992). Hence, gene flow between neighbouring Carpathian areas may have been possible at that time, and the genetic differentiation observed between the western and south-eastern Carpathian populations of *H. uniflora* would then have evolved since the end of the last glacial period (c. 14,000 years ago) due to the massive forest expansion and the isolation of high-mountain vegetation. On the other hand, this separation could be much older. Indeed, pollen and charcoal samples suggested the existence of glacial refugia for boreal forests mostly composed of conifers in the eastern Carpathians (Farcas *et al.*, 1999; Fărcaș & Tanțău, 2002; Björkman *et al.*, 2003) and adjacent Pannonian lowland (Willis *et al.*, 2000; Willis & van Andel, 2004). In the light of these fossil records, we cannot exclude the possibility that some coniferous forests survived the Last Glacial stage (Würm) in the Skhidni Beskidi Mountains (Ravazzi, 2002; Cheddadi *et al.*, 2006), thus separating alpine and subalpine vegetation zones of the western and eastern Carpathians. The strong genetic differentiation of *H. uniflora* in the Carpathians supports a long-term, pre-Würm isolation, thus rather favouring this second scenario. Because the climate was much more severe during the Riss glacial period preceding the Last Glacial stage (Würm) (Charlesworth, 1957), the rate of migration of alpine plants between the western and eastern Carpathians was probably higher during the Riss than the Würm period.

Significant genetic differentiation between populations from the eastern and southern Carpathian groups was also detected. In this part of the Carpathians, the mountain range is rather continuous, without any visible geographical barrier such as for the western/eastern Carpathian split (see above). Such eastern–southern Carpathian differentiation could be the result of geographical separation of populations during Quaternary climatic oscillations, as was shown for many high mountain taxa in the Alps (Schönswetter *et al.*, 2005). Thus, the existence of two glacial refugia in the south-eastern Carpathians is suggested.

The Apuseni population occupies a genetically intermediate position in the south-eastern Carpathian group, and it was difficult to assign either to the southern or to the eastern Carpathian cluster based on PCoA and NJ analyses (Figs 2 & 3).

In fact, the Apuseni Mountains (also known as the Bihar Mountains) occupy a particular geographical position in the Carpathians, being isolated from the high-altitude Carpathian ranges by the Transylvania Basin (Fig. 1). The mixed Apuseni population could be the remnant of a large population in the contact zone between two distinct AFLP phenotypes. In a STRUCTURE analysis (data not presented), the three plants from the Apuseni Mountains represented a mixture of two clearly separated genetic groups from the eastern and southern Carpathians.

Distribution of genetic variation across its overall distribution area

We found striking differences in within-population genetic diversity among phylogeographical groups. The highest level of variation was observed in the eastern Carpathians, followed by the western and southern Carpathians, and the Alpine populations were the least variable. Moreover, the south-eastern Carpathian group of populations harboured substantially higher numbers of rare (DW values) and region-diagnostic AFLP fragments than the populations from the western Carpathians and the Alps.

Our study included populations sampled across the whole range of *H. uniflora* (with the exception of populations growing in a very small, isolated area of the Sudetes Mountains at the northern limit of the distribution (Fig. 1). Thus, it is likely that either the Alps or the Carpathians represent the evolutionary cradle of the species. Highly variable populations characterized by high numbers of rare and region-diagnostic AFLP loci from the south-eastern Carpathians may be evolutionarily older than the populations from the Alps. Thus, evolutionarily older and more variable populations from the Carpathian territory might have colonized the Alps with subsequent reduction of genetic variation due to founder effects and/or genetic drift in marginal areas of a previously larger range. Because our data indicate a strong genetic differentiation of the populations from the Alps and the Carpathians, this colonization could have taken place earlier than the most recent post-glacial/glacial period. However, this assumption should be tested by studying phylogenetically more informative molecular data such as nuclear or plastid sequences. According to ITS phylogeny of the genus *Hypochaeris* published by Cerbah *et al.* (1998) and Samuel *et al.* (2003), *H. uniflora* forms a well-supported clade with the Eurasian species *H. maculata* L. and *H. illyrica* K. Malý, which is endemic to the Balkans. The sister species of this clade is *H. grandiflora* Ledeb., found in Asia. All these taxa belong to the same section *Achyrophorus* Scop. Thus, it seems that the geographical origin of the clade comprising *H. uniflora* could be in Asia, with possible subsequent diversification in the European mountain system. Because the Carpathians are the easternmost region where *H. uniflora* occurs, the geographical proximity of this range to Asia could have played an important role in the origin of the species.

Another possible (and possibly complementary) explanation for the low within-population variability of the Alpine populations is the occurrence of a serious genetic bottleneck. The Alps were strongly affected by repeated long-term cold periods. Large areas were covered by the ice sheet, substantially reducing the habitats suitable for the high-mountain flora and possibly reducing the population size and genetic variation. The impact of Pleistocene glaciations on the genetic structure of *H. uniflora* populations should be more significant in the Alps than in the Carpathians where only local mountain glaciers occurred.

Phylogeographical signal in the Alps and post-glacial recolonization from the eastern Alpine refugium

All 55 analysed populations of *H. uniflora* covering the entire Alps formed a genetically homogeneous group. Although rarely observed (Schönswetter *et al.*, 2005), such a lack of phylogeographical differentiation of Alpine populations was found in *Oxytropis campestris* s.l. (Schönswetter *et al.*, 2004a) and *Saxifraga oppositifolia* (Holderegger *et al.*, 2002). The unresolved phylogeographical pattern of *O. campestris* s.l. was explained by recent post-glacial colonization of the Alps from unknown refugia (Schönswetter *et al.*, 2004a). A similar scenario of post-glacial immigration to the Alps was proposed for the arctic-alpine species *Saxifraga oppositifolia*. Holderegger *et al.* (2002) pointed out that even if relict populations of *S. oppositifolia* survived the ice age *in situ* in Alpine nunataks, they were swamped by massive immigration of periglacial genotypes of unknown geographical origin after the ice retreated.

Despite the absence of a clear phylogeographical pattern within *H. uniflora* in the Alps, there were some trends indicating that the eastern Alps may have been a glacial refugium from which *H. uniflora* colonized the deglaciated Alpine areas. Most populations from the eastern Alps were more variable and exhibited a higher proportion of rare AFLP fragments (higher DW values) than those from the central and western Alps. Moreover, all indices of within-population diversity (AFLP band richness, DW values, Jaccard index, percentage of polymorphic loci) were significantly correlated with longitude (Fig. 4), indicating the gradual loss of genetic diversity from east to west. The eastern Alpine populations nos 49–55, forming a slightly separated group in the PCoA, were sampled in localities within the large putative refugium of silicolous plants in the eastern Alps (cf. Tribsch & Schönswetter, 2003; Schönswetter *et al.*, 2005). Thus, our data support the hypothesis of the existence of an eastern Alpine refugium for the subalpine taxon *H. uniflora*. The patterns of genetic diversity allow us to suggest that these populations from the eastern Alps served as a gene pool for post-glacial colonization of the Alps. All within-population parameters of diversity strongly indicate that the colonization process was rapid and accompanied by a loss of genetic diversity through multiple founder effects, which is in agreement with theoretical models of population genetics (Nei *et al.*, 1975; but see also Comps *et al.*, 2001). In addition to an eastern Alpine peripheral refugium, we cannot exclude the possibility that small isolated

populations also survived along the southern border of the Alps, as shown by the relatively high indices of genetic variation in southern peripheral populations. However, if they existed, they were probably absorbed by massive migration of the eastern Alpine genotypes, as shown for *Saxifraga oppositifolia* (Holderegger *et al.*, 2002).

Concluding remarks and perspectives

The genetic structure of *H. uniflora* revealed the existence of at least three geographically separated lineages, suggesting the long-term isolation of populations from the Alps and the western and the south-eastern Carpathians. The very detailed and spatially even sampling enabled us to detect a genetic barrier between western and south-eastern Carpathian populations, in agreement with previous floristic studies documenting this discontinuity. To our knowledge, this is the first evidence for such a barrier in the Carpathians based on molecular data in vascular plants.

From the biogeographical point of view, a strong genetic differentiation between the Alps and the Carpathians is noteworthy. This finding can be linked with the possible region of origin of *H. uniflora* (Carpathians), and/or with a strong reduction of genetic diversity in the Alps during the Last Glacial period. In this light, it would be interesting to infer and compare the phylogeographical history of other closely related *Hypochaeris* taxa.

Previous studies focusing on Alpine phylogeography usually identified two or more glacial refugia for (sub)alpine plants in the Alps (Schönswetter *et al.*, 2005). In contrast, we found no clear geographical structure at the population level in *H. uniflora*. However, the clinal pattern of population diversity strongly suggests the existence of an eastern Alpine refugium, from which the rest of the Alps was probably colonized after the retreat of the ice sheet.

The sampling strategy used in the present study allowed us to infer the phylogeographical patterns of *H. uniflora* and seems to be a promising design for future phylogeographical studies. Although only three plants from each population were analysed genetically, this was counter-balanced by the possibility of evenly sampling many populations across the distribution area. However, we suggest sampling and analysing more than three individuals per populations (four to five) in order to avoid a possible exclusion of population(s) due to problematic individuals (e.g. unsuccessful AFLP procedure).

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BIOSKETCHES

Patrik Mráz is currently a Marie Curie fellow at LECA in Grenoble, and works on the phylogeography of an arcto-alpine, sexual-apomictic *Hieracium alpinum* s.str. He is interested mainly in taxonomy, hybridization and the processes involved in the microevolution of the polyploid genera of *Hieracium* and *Pilosella*.

Myriam Gaudeul is now a researcher at the Muséum National d'Histoire Naturelle (Paris, France) where she investigates aspects of plant evolution including biogeography, adaptation and speciation.

Delphine Rioux and **Ludovic Gjelly** are interested in the application of molecular methods in phylogenetic and ecological studies.

Philippe Choler is a plant ecologist. His main scientific interests concern the functional and evolutionary ecology of alpine plants.

Pierre Taberlet is the Director of the Laboratoire d'Ecologie Alpine, and his interests focus on the conservation genetics and molecular ecology of different plant and animal species.

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