

Phylogeography of subalpine tall-herb species in Central Europe: the case of *Cicerbita alpina*

Fylogeografie *Cicerbita alpina*, druhu středoevropských subalpínských společenstev

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Cicerbita alpina was selected to elucidate the phylogeography of tall-herb species, an ecological group whose Quaternary history is rarely addressed. This species is a typical component of subalpine herbaceous communities in the mountains of Europe. Samples collected for this study comprised the entire range of species, with a focus on those in the Carpathians. The analysis based on AFLP fingerprinting revealed a lack of a strong phylogeographical structure implying that the different parts of the present-day range have not been isolated for a long period of time probably due to the biological characteristics of the species, such as its ability to disperse over great distances. However, the genetic structure indicates some phylogeographical trends, which may reflect traces of survival in local refugia and subsequent diversification into separate lineages during the last glacial period. Within the Carpathians, the division into the Western and South-Eastern Carpathian population groups is apparent. This division is maintained at a larger scale. In particular, the South-Eastern Carpathian group is similar to the Balkan populations, while the Western Carpathian populations are closely related to those in the Eastern Alps and Sudetes. The Scandinavian populations also have a genetic affinity with the latter group and originated from a source in the Eastern Alps or Western Carpathians, presumably via a stepping stone in a northern refugium.

Key words: AFLP, Carpathians, *Cicerbita alpina*, genetic diversity, European mountain system, refugia, tall-herb communities

Introduction

The mountains of Europe constitute the European Alpine system (sensu Ozenda 1985), in which each high mountain range represents a terrestrial “island” surrounded by low-altitude environments characterized by present-day climatic conditions unfavourable for mountain plants (Birks & Willis 2008, Kropf et al. 2008). The modern mountain flora inhabiting these “islands” has been strongly influenced by climatic changes, which took place during the Quaternary (e.g. Szafer 1964, Lang 1994, Hewitt 1996, Comes & Kadereit 1998, 2003, Stehlik 2000, Kadereit et al. 2004). Paleobotanical and recent phylogeographic studies provide valuable insights into the abilities of plants to survive and recolonize available areas (e.g. those exposed by the retreat of glaciers). The present-day areas of southern Europe with the highest floristic richness are recognized as the main refugia for European biota (Konnert & Bergmann 1995, Demesure et al. 1996, Dumolin-Lapègue et al. 1997, Taberlet et al. 1998, Newton et al. 1999, Tzedakis 2004). In the case of mountain plants, recent research provides information on the existence of local refugia located in different European mountain areas, which confirm previous hypotheses about

their existence put forward on the basis of floristic and paleobotanical findings (e.g. Pawłowski 1970, Obidowicz 1996, Vargas 2003, Schönswetter et al. 2005, Schmitt 2009, Ronikier 2011). Recently, it was stressed that cryptic refugia were important for the survival and migration of the flora. They are situated in the lowland parts of northern, Central and eastern Europe (Bhagwat & Willis 2008, Birks & Willis 2008). This hypothesis is in line with palynological data on the survival of tree species in Central and Central-eastern Europe (Ralska-Jasiewiczowa et al. 2004, Willis & van Andel 2004, Cheddadi et al. 2006). The studies published to date indicate different, species-specific abilities of surviving glaciations during the Quaternary climatic oscillation, which depend among others on biological characteristics of the species. There are several possible explanations of the present-day distributions of alpine and arctic-alpine species, such as migration from southern refugia (e.g. Vargas 2003, Kropf et al. 2008, Csergö et al. 2009), migration from local refugia (e.g. Schönswetter et al. 2005, Mráz et al. 2007, Ronikier et al. 2008a), long-distance dispersal (e.g. Kropf et al. 2003) and in situ survival (e.g. Stehlik et al. 2002, Bettin et al. 2007).

Subalpine species constitute a distinct ecological group of mountain plants. While their geographical distributions can overlap those of alpine species, their altitudinal range may be much wider than that of alpine species. There are only a few phylogeographical studies of subalpine or montane plant species in Central Europe: *Hypochoeris uniflora* (Mráz et al. 2007), *Polygonatum verticillatum* (Kramp et al. 2009), *Rosa pendulina* (Fér et al. 2007), *Trollius europaeus* s.l. (Després et al. 2002) and recently *Cicerbita alpina* (Michl et al. 2010). Among the subalpine species, tall herbaceous species constitute a highly habitat-specific group. They build tall-herb communities in special edaphic and microclimatic conditions, mainly in fertile and humid habitats along streams and in the vicinity of springs above the upper forest limit (Pawłowski et al. 1928, Pawłowski 1966). With reference to syntaxonomy, tall-herb communities are classified within the class *Mulgedio-Aconitetea* Hadač et Klika in Klika 1948. In the floristic composition of communities, there are tall dicotyledonous perennials, including species characteristic of the alliance *Adenostylion*, e.g. *Adenostyles alliariae*, *Cicerbita alpina* and *Doronicum austriacum* (Kliment et al. 2007, Stachurska-Swakoń 2008, 2009a). Flora of tall-herb communities includes different chorological elements (Stachurska-Swakoń 2007, 2009a, b, Stachurska-Swakoń & Kuž 2011).

This paper is dedicated to one of the main tall-herb species, *Cicerbita alpina*, the range of which covers the mountains of Central and southern Europe and Scandinavia. Recently, Michl et al. (2010) published a phylogeographical study carried out in a western, mainly Alpine part of its geographical distribution. Their study indicates the distinctiveness of the Pyrenean and southern Alpine populations, which as suggested, may be a result of survival in these regions during the Würm glaciation. They also reveal that the genetic structure of this species from the Alps to Scandinavia is relatively uniform and support the idea of the importance of northern ('cryptic') refugia for the survival of this species during this glaciation. Two other articles on this species, based on fewer samples (pilot studies for larger phylogeographical hypotheses), discuss local problems: division of the Western and Southern Alpine populations (Michl et al. 2007) and genetic impoverishment of the island-like populations at the northernmost locality in the Carpathians (Stachurska-Swakoń et al. 2011). In the present study, we aim to determine which of the proposed scenarios on the origin of the present-day mountain flora applies to subalpine tall-herb species in Central Europe. The research focused on the Carpathians; the significance of this

mountain system in the development of the modern richness of the vegetation cover is emphasized in many phytogeographical and recently also in phylogeographic papers (e.g. Pawłowski 1969, 1970, Boşcaiu & Täuber 1985, Mráz et al. 2007, Puşcaş et al. 2008, Ronikier 2011). This paper aims to unravel the phylogeographical pattern of *Cicerbita alpina* as a typical tall-herb species and test the assumption that ecological (large altitudinal range) and biological (ability to disperse over great distances) features prevent a strong genetic differentiation of tall-herb species populations within the Carpathians (taking into account their topographically isolated western, eastern and southern parts) and across the separate mountain ranges in Central Europe.

Materials and methods

The study species

Cicerbita alpina (L.) Wallr. (*Asteraceae*, *Lactucoideae*, *Lactuceae*) is one of the four representatives of the genus native to the mountains of Europe. It has a disjunct distribution, which includes the Alps, northern part of the Balkans, Carpathians, Sudetes, Scandinavia and the lower mountains of Central Europe (Jura Mts, Black Forest, Harz Mts and Erz Mts). In addition it is rare in the Pyrenees and Scotland (Hultén & Fries 1986, Marren et al. 1986, Meusel & Jäger 1992, Alexander 2006). It is a characteristic species of tall-herb communities of the *Adenostylon alliariae* alliance, occurring principally in the subalpine altitudinal zone (Pawłowski 1966, Oberdorfer 1983, Stachurska-Swakoń 2009a). It always grows in fertile and humid habitats, so it may also be found outside typical tall-herb associations, in moist forests, source areas of rivers and along banks of streams (Benum 1958, Tacik 1972, Oberdorfer 1983, Lid 1987). In mountains, it grows between 700 m (rarely below) and 2000 m a.s.l.

Cicerbita alpina is a simple or branched, rhizomatous perennial herbaceous plant, 50–250 cm tall, with numerous capitula of pale blue ligules. An involucre is usually 10–15 × 7–10 mm. It is an insect pollinated species and probably self-incompatible, as is common in *Asteraceae* (Richards 1997). Long-distance dispersal by wind is possible as the achenes (4.4–5.0 mm) have a pappus consisting of two rows of simple hairs (Tacik 1972, Sell 1997). Only diploids with $2n = 18$ are known (Skalińska & Pogan 1966, Májovský et al. 1987, Doležalová et al. 2002).

Sampling

Forty-two populations of *Cicerbita alpina* were sampled in various parts of the species' geographical distribution (Table 1, Fig. 1). The sampling focused on the Carpathians; 27 populations were studied from this area, covering all the main parts of the range (Western, Eastern and Southern Carpathians), represented by 16, 7 and 4 populations, respectively. Samples were also collected from populations in neighbouring ranges: the Alps (5 populations), Balkans (3), Dinaric Mts (1), Scandinavia (2) and the Sudetes (4). Five to 10 plants were randomly sampled from each population. Fragments of fresh leaves were dried and stored in hermetically sealed tubes filled with silica gel. In total, 226 samples (2–8 per population) were included in the analysis.

Table 1. Geographical origin and genetic characteristics of the populations of *Cicerbita alpina* sampled based on AFLP markers. Localities from which the samples were collected are given. Country: A – Austria, BG – Bulgaria, CH – Switzerland, NO – Norway, PL – Poland, RO – Romania, SK – Slovakia, SL – Slovenia, UA – Ukraine. Alt. – altitude (m a.s.l.). N – number of samples, % poly – percentage of polymorphic bands, Nei's D (L; U) – Nei's gene diversity with lower and upper band of 95% confidence intervals, DW – frequency-down-weighted marker values, M_{priv} – private markers.

No	Code	Locality	Region/country	Coordinates	Alt.	N	% poly	Nei's D (L; U)	DW	M _{priv}
1	A	Swiss Jura, Col du Marchairuz	W Alps/CH	N46.32 E6.14	1350	6	29.7	0.13 (0.11; 0.16)	5.67	1
2	AA	Steiermark, Eisenerzer Alpen	E Alps/A	N47.28 E14.33	1200	5	29.7	0.15 (0.12; 0.18)	5.59	3
3	AB	Swiss Alps, Oberalp Pass	W Alps/CH	N46.34 E8.20	2000	6	12.0	0.06 (0.04; 0.08)	7.94	2
4	AL	Dinaric Alps, Mt Snežnik	Dinaric Alps/SL	N45.35 E14.26	1670	4	28.4	0.16 (0.12; 0.19)	3.57	2
5	AS	Kamniško-Savinjske Alpe, Storžič	E Alps/SL	N46.20 E14.25	1400	5	36.6	0.18 (0.15; 0.22)	5.11	2
6	B	Babia Góra	W Carpathians/PL	N49.34 E19.31	1310	8	44.0	0.19 (0.16; 0.23)	8.66	1
7	BH	Rodopi, Malak Perelik	Balkans/BG	N41.36 E24.33	2060	3	21.4	0.14 (0.11; 0.18)	2.84	4
8	BP	Pirin, Vihren hut	Balkans/BG	N41.46 E23.25	1740	5	21.0	0.10 (0.08; 0.13)	6.28	2
9	BR	Rila, the Maliovisa valley	Balkans/BG	N42.11 E23.22	1700	5	20.5	0.10 (0.07; 0.13)	5.29	4
10	F	Massif des Bauges, Pécloz	W Alps/F	N45.37 E6.13	1602	6	24.6	0.12 (0.09; 0.15)	6.80	1
11	GA	Gorce	W Carpathians/PL	N49.35 E20.12	920	6	44.6	0.20 (0.16; 0.23)	5.68	1
12	I	Góry Bialskie	Sudetes/PL	N50.16 E17.00	675	6	33.0	0.15 (0.12; 0.18)	5.54	1
13	K	Karkonosze, Mały Śnieżny Kocioł	Sudetes/PL	N50.47 E15.33	1250	5	39.0	0.20 (0.17; 0.23)	5.14	4
14	KB	Karkonosze, Śnieżka	Sudetes/PL	N50.43 E15.44	1230	5	26.0	0.13 (0.10; 0.16)	4.90	2
15	NS	Finnmark county, Elvenes	Scandinavia/NO	N69.50 E29.30	26	6	33.5	0.04 (0.02; 0.06)	5.49	1
16	NT	Hedmark county, Gammeldalen	Scandinavia/NO	N62.03 E12.13	710	6	18.6	0.08 (0.06; 0.11)	7.21	0
17	PM	Beskid Mały, Jaworzynka	W Carpathians/PL	N49.47 E19.26	846	5	9.8	0.05 (0.03; 0.07)	5.30	0
18	PN	Beskid Mały, Rzyki	W Carpathians/PL	N49.46 E19.23	567	5	10.2	0.05 (0.03; 0.07)	5.59	1
19	PW	Beskid Wyspowy, Mogielica	W Carpathians/PL	N49.39 E20.16	1042	5	19.5	0.09 (0.07; 0.12)	5.93	1
20	PZ	Beskid Żywiecki, Potok Cebulowy	W Carpathians/PL	N49.33 E19.16	865	5	33.5	0.17 (0.13; 0.20)	5.01	2
21	R	Beskid Żywiecki, Wielka Racza	W Carpathians/PL	N49.24 E18.58	1020	5	29.3	0.14 (0.11; 0.17)	5.09	0
22	RB	Munții Bucegi, Valea Jepilor	S Carpathians/RO	N45.23 E25.30	1350	8	37.7	0.16 (0.13; 0.19)	8.23	0
23	RC	Munții Ceahlău, Ocolașul Mare	E Carpathians/RO	N46.56 E25.57	1580	5	27.4	0.14 (0.11; 0.17)	4.67	0
24	RF	Munții Făgărașului, Valea Sămbata	S Carpathians/RO	N45.37 E24.48	1300	8	43.2	0.17 (0.15; 0.20)	6.80	0
25	RO	Munții Rodnei, Pietrosu Mare	E Carpathians/RO	N47.35 E24.38	2000	5	30.7	0.15 (0.12; 0.18)	4.62	1
26	RR	Retezat, Bucura	S Carpathians/RO	N45.36 E22.86	1860	4	33.5	0.18 (0.15; 0.22)	3.90	0
27	RT	Retezat, Radeșu Mare	S Carpathians/RO	N45.21 E22.46	1500	5	22.3	0.11 (0.08; 0.13)	4.24	0
28	S	Śnieżnik	Sudetes/PL	N50.13 E16.50	822	6	38.4	0.18 (0.15; 0.21)	5.33	0
29	SB	Belianske Tatry	W Carpathians/SK	N49.14 E20.13	1700	2	18.6	0.19 (0.14; 0.24)	1.81	–
30	SF	Malá Fatra, Chleb	W Carpathians/SK	N49.11 E19.03	1480	7	26.0	0.10 (0.08; 0.13)	6.98	1
31	SN	Nízke Tatry, Sedlo pod Veľ'kým Bokom	W Carpathians/SK	N48.55 E19.52	1480	3	25.1	0.17 (0.13; 0.21)	3.06	2

No	Code	Locality	Region/country	Coordinates	Alt.	N	% poly	Nei's D (L; U)	DW	M _{priv}
32	SS	Vysoké Tatry, Štrbské Pléso	W Carpathians/SK	N49.07 E20.03	1359	6	37.2	0.16 (0.14; 0.19)	5.98	0
33	ST	Západné Tatry, Roháče	W Carpathians/SK	N49.12 E19.44	1580	5	31.6	0.16 (0.13; 0.20)	4.49	1
34	SZ	Vysoké Tatry, Kriváň	W Carpathians/SK	N49.09 E19.59	1580	5	32.1	0.15 (0.12; 0.19)	5.11	1
35	TG	Tatry Wysokie, H. Gąsienicowa	W Carpathians/PL	N49.14 E20.01	1476	6	36.7	0.17 (0.14; 0.20)	5.82	0
36	TK	Tatry Zachodnie, Dol. Kościeliska	W Carpathians/PL	N49.14 E19.51	980	6	22.3	0.10 (0.08; 0.13)	6.43	0
37	TM	Tatry Wysokie, Morskie Oko	W Carpathians/PL	N49.11 E20.02	1401	6	33.5	0.16 (0.13; 0.18)	5.45	0
38	U	Gorgany, Berezovacky Klyvky	E Carpathians/UA	N48.46 E24.31	1250	4	34.9	0.21 (0.18; 0.25)	3.48	2
39	UB	Chornohora, Zaroslak	E Carpathians/UA	N48.16 E24.53	1385	5	28.4	0.12 (0.09; 0.15)	4.96	0
40	UC	Chornohora, Cybulnik	E Carpathians/UA	N48.15 E24.32	1330	7	43.0	0.17 (0.14; 0.19)	6.84	0
41	UH	Chornohora, Howerla	E Carpathians/UA	N48.15 E24.30	1500	5	12.0	0.06 (0.04; 0.09)	9.08	1
42	UO	Chornohora, Zaroslak	E Carpathians/UA	N48.16 E24.53	1520	6	35.8	0.16 (0.13; 0.19)	6.74	1

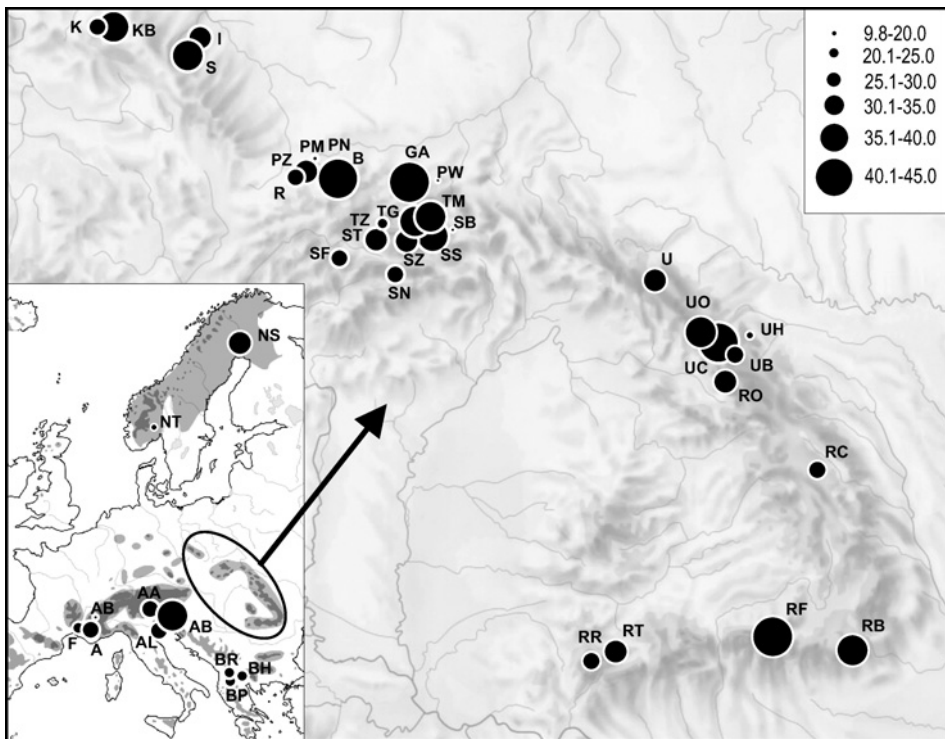


Fig. 1. – Localities of the populations of *Cicerbita alpina* sampled. The geographical distribution of the species (grey shading) is based on Hultén & Fries (1986) and Lid (1987). Codes of populations are given in Table 1. Size of circle indicates the percentage of polymorphic loci.

DNA extraction and AFLP analyses

Total DNA was extracted from ca 15 mg of dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen) and following the manufacturer's protocol. The final elution step was carried out using $2 \times 50 \mu\text{L}$ elution buffer. Concentration and quality of the extracted DNA were estimated against a λ -DNA concentration gradient on 1% agarose gel stained with ethidium bromide. AFLP reproducibility tests (Bonin et al. 2004) included within-plate ($n = 12$) and between plate ($n = 9$) replicates independently analysed from DNA extracts.

AFLP analysis was carried out following the methods of Vos et al. (1995) with modifications, as described in detail by Ronikier et al. (2008a). Double-digestion of DNA was performed using EcoRI and MseI enzymes. A preliminary screening of 12 selective primer pair combinations on four samples from geographically distant populations was done. Three pairs of selective primers were selected based on polymorphism, clarity and reproducibility of AFLP profiles: EcoRI-ACC/MseI-CAG, EcoRI-ACA/MseI-CTG, and EcoRI-AGG/MseI-CTC. All EcoRI selective primers were 5'-fluorescent-labelled (6-FAM). 20-fold diluted selective amplification products were separated with GeneScan-500 ROX (Applied Biosystems) internal size standard, using 36 cm capillaries and POP 4 polymer on an ABI PRISM 3100-*Avant* sequencer (Applied Biosystems).

Data analysis

AFLP fragments were manually scored in the size range of 50–500 bp using the software GeneScan 3.7 (Applied Biosystems) and Genographer 1.6 and assembled in a binary presence/absence matrix. Basic diversity statistics were calculated for each population: total number of AFLP bands, percentage of polymorphic markers and Nei's gene diversity in populations (Nei 1987). Nei's gene diversity was computed with confidence intervals and standard deviation to estimate the consistency of values. Frequency-down-weighted marker values DW (Schönewetter & Tribsch 2005) were used as a standardized measure of divergence and identification of long-term isolation. Categories of markers showing the genetic uniqueness of populations were observed: discriminating markers, defined as those present in all analyzed samples of a respective population and absent elsewhere, and other private fragments unique to particular populations but not fixed in all samples. In addition, genetic similarities between populations as Nei's Original Measures of Genetic Identity were used to estimate the genetic affinities between the populations studied (Nei 1978). Diversity statistics were computed using software POPGENE ver. 1.32 (Yeh et al. 1997) and AFLPdat (Ehrich 2006). Diversity statistics were also calculated for geographical groups defined a priori that followed the main physiographically distinct massifs: Eastern Alps, Western Alps, Balkans, Eastern Carpathians, Southern Carpathians, Western Carpathians, Sudetes and Scandinavia.

The possible correlation between geographical location and within-population genetic variation was tested using Pearson correlation coefficients and the differences between genetic diversity parameters in geographical groups estimated using a Kruskal-Wallis rank sum test (both computed with STATISTICA 9.0 software). The Mantel test was carried out to look for significant correlations between the pair wise genetic and geographic distances using a permutation procedure (1000 permutation) in ARLEQUIN 3.1 (Excoffier et al. 2005).

The relationships between individuals and the presence of genetic groups were analyzed using several methods: principal coordinate analysis (PCoA) based on inter-individual Jaccard distances was computed using SYN-TAX 2000 (Podani 2001). An unrooted neighbour-joining (NJ) dendrogram based on the genetic distance of Nei & Li was generated and bootstrapped using 1000 replicates with the software FAMD (Schlüter & Harris 2006). Bayesian non-hierarchical clustering of individuals was applied using STRUCTURE 2.2.3 (Falush et al. 2007), based on an ad-mixture model with correlated allele frequencies. The numbers of K from 2 to 10 were tested with 10 replicates per K. 1×10^6 Markov Chain Monte Carlo repetitions were applied with a burn-in period of 200 000. Outputs of all STRUCTURE runs were analyzed using the R-script Structure-sum (Ehrich 2006). Average similarity coefficients among runs were calculated for each K to verify the consistency of replicated runs. The following values were observed in order to assess the most appropriate number of clusters: (i) the $\ln P(D)$ values, estimates of posterior probabilities provided in STRUCTURE outputs, examined as a function of increasing K; (ii) ΔK values, estimating the change in the likelihood function with respect to K and estimated as an indicator of the most reliable clustering structure (Evanno et al. 2005). PCoA, NJ and Bayesian analysis were performed on the complete data set and also only on the Carpathian individuals.

Analyses of molecular variance (AMOVA) within and between populations were performed using ARLEQUIN 3.1 (Excoffier et al. 2005) to test the partitioning of genetic variation within and among populations, as well as the importance of the main groups identified by cluster analyses.

Results

Genetic diversity within- and among-populations

AFLP analysis resulted in 215 markers; 161 of them (75%) were polymorphic across the data set. Data quality tests indicated a high reliability of the AFLP bands with an overall repeatability of above 98%. The individual number of markers ranged from 97 (NS_5, Scandinavia) to 159 (TK_2, Western Carpathians). The average number of markers per population varied from 119 (TG, Western Carpathians) to 154 (TK, Western Carpathians) with a mean of 143.7 (SD = 7.16). The percentage of polymorphic markers varied from 9.8% (PM, Western Carpathians) to 44.6% (GA, Western Carpathians) with a mean of 29.35% (SD = 8.89) (Table 1, Fig. 1). For the whole data set, Nei's gene diversity varied from 0.04 (NS, Scandinavia) to 0.21 (U, Eastern Carpathians) with a mean of 0.14 (SD = 0.04). The DW coefficient ranged from 1.81 (SB, Western Carpathians) to 9.08 (UH, Eastern Carpathians) with a mean of 5.54 (SD = 1.5). No discriminating bands were found in the populations studied. Private bands occurred in 26 populations; the highest number, four per population, was in the Balkan (BH, BR) and Sudetes (K) populations (Table 1). Apart from those population-specific bands, only a few private bands were found in geographical groups: two in the Balkan, Southern Carpathian and Scandinavian population groups and one in the Western Carpathian population group.

The index of the genetic similarity between populations – Nei's Original Measure of Genetic Identity – varied from 0.77 (between AB population from the Western Alps and PN from the Western Carpathians) to 0.96 (between RB and RO both from the Southern

Carpathians) with a mean of 0.89 (SD = 0.03). The highest values of this measure characterized the Southern Carpathian populations (from 0.93 to 0.96). The lowest values were recorded in the AB population in the Western Alps (with a mean of 0.88) and PM and PN in the Western Carpathians (with a mean of 0.86).

The Mantel test computed for the whole data set revealed no significant correlation between the genetic and geographic distances ($r_m = 0.391$, $P = 0.269$). A weak correlation was found for the Carpathian populations ($r_m = 0.168$, $P = 0.0341$).

The differences in genetic statistics between defined geographical groups (a Kruskal-Wallis rank sum test) estimated for the whole data set and separately for the Carpathian populations were not statistically significant. The correlations between geographic distances and genetic diversity indices (Pearson correlation coefficient) were also not statistically significant, however, logarithmic transformations yield a significant correlation between longitude and Nei's gene diversity ($r = -0.374$, $P = 0.0147$).

Inference of genetic groups and relationships among populations

The neighbour-joining analysis revealed little structure in the data set and the bootstrap support for larger groups was below 50% (Fig. 2). However, individuals formed several clusters, the most distinct of which comprised individuals from the Eastern and the Southern Carpathians and Balkans. Individuals from the Western Carpathians were heterogeneous and grouped partly in a separate cluster, partly in another one together with most of the Sudetes populations (I, K, S) and also contributed to a small cluster with the remaining individuals from the Sudetes (KB) and the Eastern Alps (AS). The remainder of the tree included diverse small clusters with, in particular, populations from the Western Alps, Scandinavia and Dinaric Mts.

The PCoA ordination analysis confirmed the weak structuring of the data set. There is a slight division along the first axis between the Eastern and Southern Carpathian with the Balkan individuals and the rest of the individuals (Fig. 3). Additionally, the distribution of individuals from the Western Alps in the PCoA scatter diagram, upper right side, only slightly overlap that of individuals from other geographical groups.

The results of the Bayesian STRUCTURE clustering were congruent with the inference based on clustering and ordination methods (Fig. 4A). The mean L (K) and ΔK values indicated $K = 7$ as the most appropriate clustering for the whole data set. The average similarity coefficients among runs for $K = 7$ was 0.99 (SD = 0.002). Such high similarity coefficients characterized also $K = 2$ and 3, which however had lower values of other indicators. Other K values were characterized by lower similarity coefficients (from 0.5 to 0.93). Seven STRUCTURE clusters partly followed the geographical groups: the Eastern Alps (with the Dinaric Mts and a number of individuals from the Western Carpathian and Sudetes), the Western Alps, the Western Carpathians (with five individuals from the Sudetes S population), Scandinavia and the Southern Carpathians with individuals from the Eastern Carpathians and Balkans. A small cluster contained some of the Sudetes individuals (population I and partly S) and five individuals from the Western Carpathians (populations GA, SB). The last cluster consisted of a few individuals from different mountain ranges across this species geographical distribution. There was a high level of admixture in some of the populations: the Sudetes (K, KB, S), Eastern Alps (AS), Dinaric Mts (AL), Southern Carpathians (RF, RT) and Western Carpathians (B, SB).

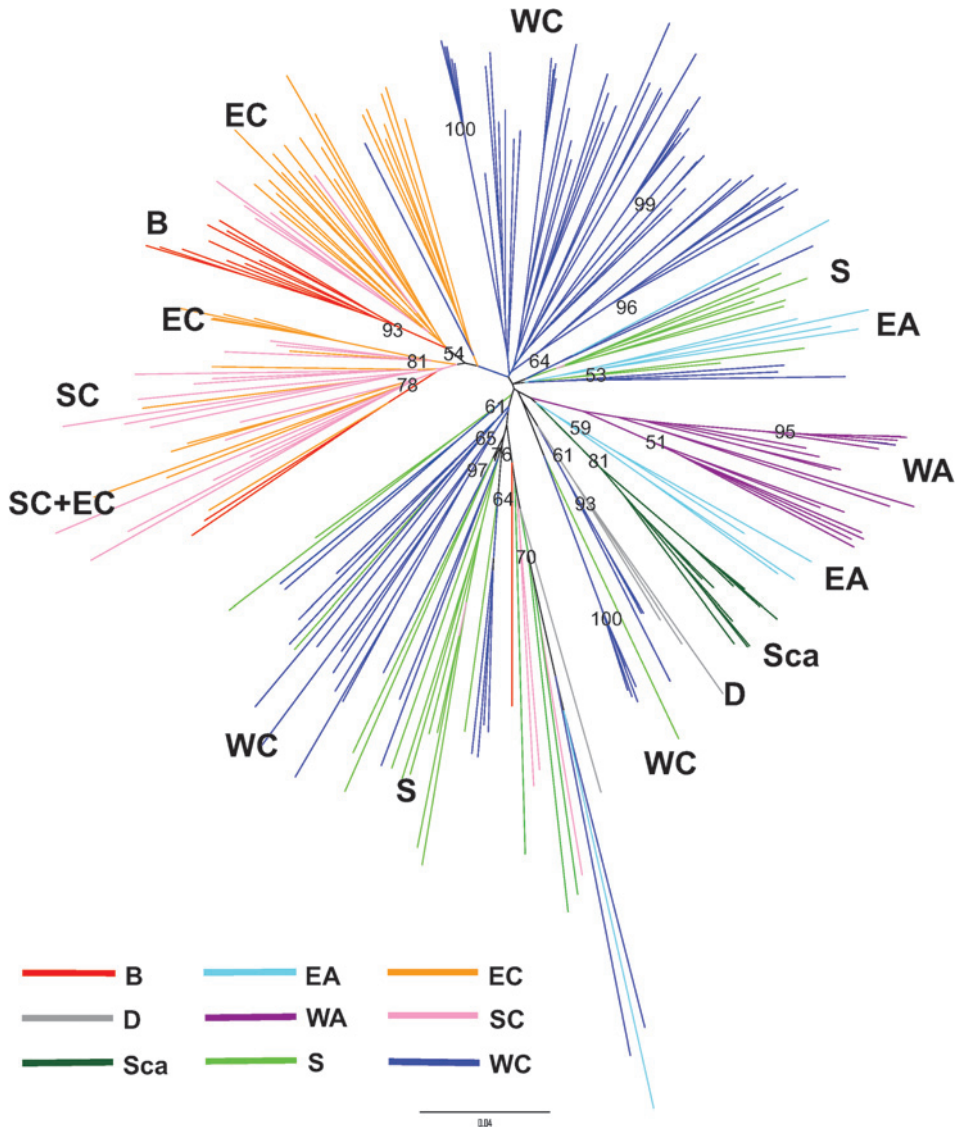


Fig. 2. – Unrooted neighbour-joining tree of *Cicerbita alpina* individuals based on Nei & Li coefficient. Bootstrap values above 50% are given at nodes. Codes of populations as in Table 1. Geographical regions: B – Balkans, EA – Eastern Alps, WA – Western Alps, EC – Eastern Carpathians, SC – Southern Carpathians, WC – Western Carpathians, D – Dinaric Mts, S – Sudetes, Sca – Scandinavia.

Analyses performed separately for the Carpathian populations indicated divisions in line with the geographical distribution of samples. Both PCoA and Bayesian analyses showed affinities between populations from the Southern and Eastern Carpathians, whereas populations from the Western Carpathians were distinctly separated (Figs 4B, 5). The PCoA indicated a subgroup within the Western Carpathians formed by three populations situated at low altitudes in a somewhat island-like situation at the periphery of the

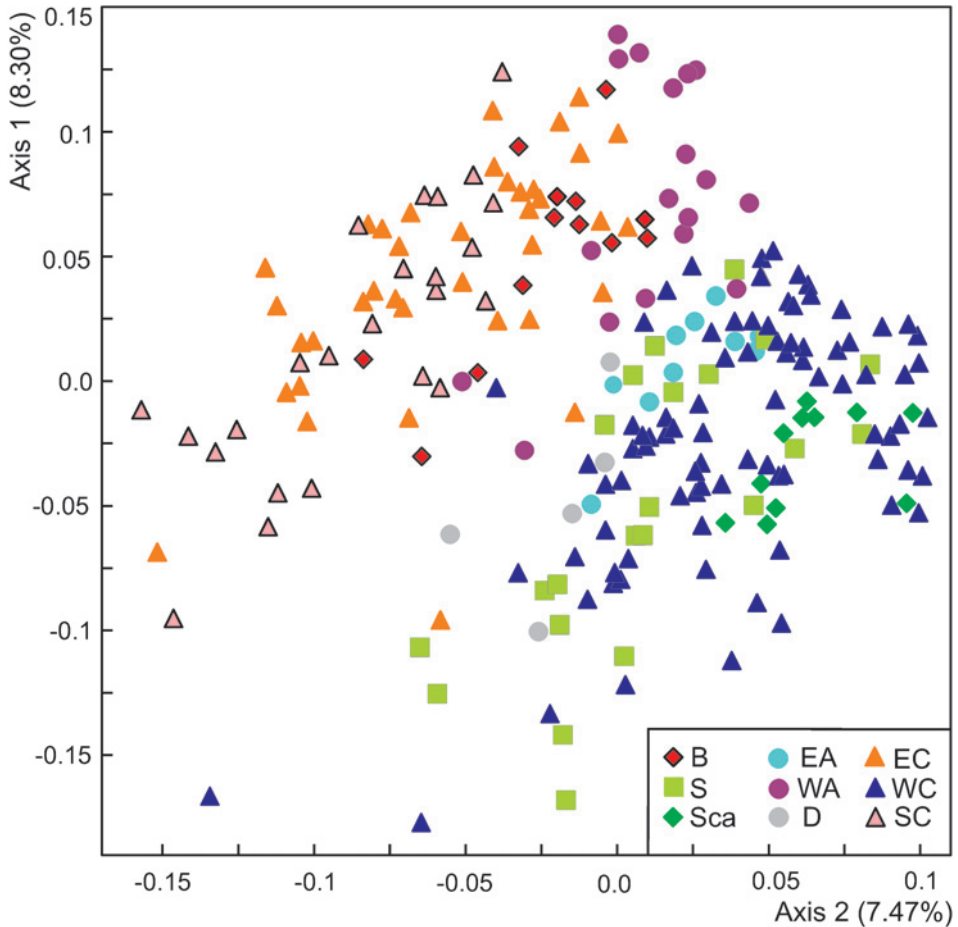


Fig. 3. – Principal coordinate analysis (PCoA) of *Cicerbita alpina* individuals based on Jaccard coefficient of AFLP phenotypes. B – Balkans, EA – Eastern Alps, WA – Western Alps, EC – Eastern Carpathians, SC – Southern Carpathians, WC – Western Carpathians, D – Dinaric Mts, S – Sudetes, Sca – Scandinavia.

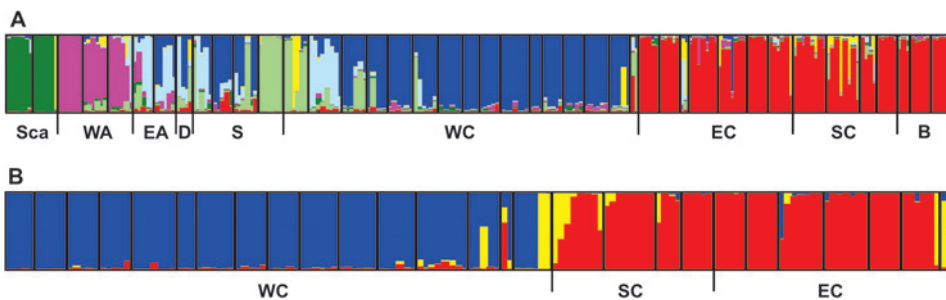


Fig. 4. – Bayesian admixture analysis of the populations of *Cicerbita alpina* using STRUCTURE software. A – analysis of complete data set for $K = 7$; B – analysis of the Carpathian individuals for $K = 3$. Lines separate geographical groups: B – Balkans, EA – Eastern Alps, WA – Western Alps, EC – Eastern Carpathians, SC – Southern Carpathians, WC – Western Carpathians, D – Dinaric Mts, S – Sudetes, Sca – Scandinavia.

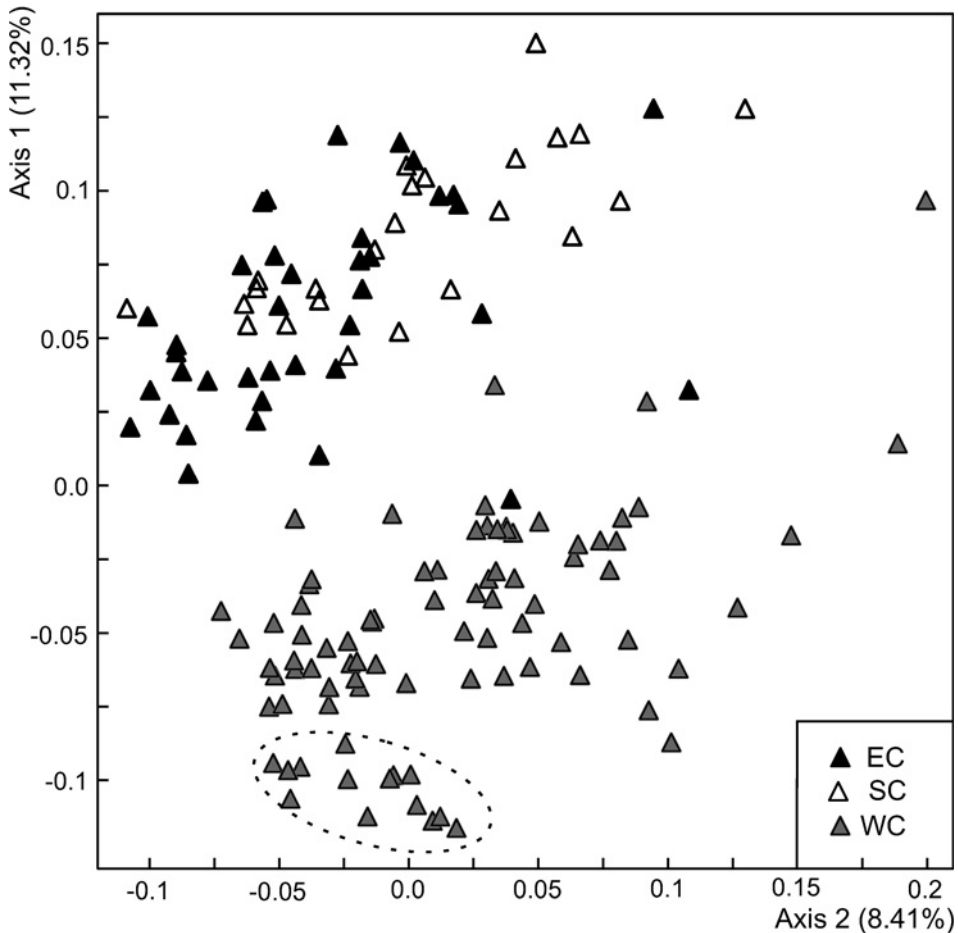


Fig. 5. – Principal coordinate analysis (PCoA) of the Carpathian individuals of *Cicerbita alpina* based on Jaccard coefficient of AFLP phenotypes (27 populations, 147 samples). EC – Eastern Carpathians, SC – Southern Carpathians, WC – Western Carpathians; dash line delimits populations PM, PN and SF – see text for explanation.

Western Carpathians (PM, PN, SF; lower part in Fig. 5). This subgroup was also confirmed in the neighbour-joining tree of the Carpathian samples (data not shown). The Bayesian analysis with $K = 3$ (with the highest value of ΔK , $L(K)$ and an average similarity coefficient of 1.0 characterizing only this K value) additionally shows a third cluster containing a few individuals from different populations (the Western Carpathians: GA, TM, Eastern Carpathians: UO, Southern Carpathians: RF).

Non-hierarchical AMOVA for all the individuals studied attributed 30.5% of the genetic variability between populations and 69.5% within populations ($F_{ST} = 0.31$; Table 2). When applying the division into a priori defined geographical groups (i.e. Eastern Alps, Western Alps, Eastern Carpathians, Southern Carpathians, Western Carpathians, Balkans, Scandinavia, Sudetes), 12.6% of the variability was observed between the groups and 20.7% between populations within the groups. Genetic variation among selected geographical groups based on F_{ST} values indicated the highest differences for the Balkan vs.

Scandinavian populations ($F_{ST} = 0.51$) and the Balkan vs. Western Alpine populations ($F_{ST} = 0.49$). Low F_{ST} values and small contribution of the variability between the groups was found for the Eastern Alps, Sudetes and Western Carpathians (2.8% between the groups; $F_{ST} = 0.28$). For the Carpathian populations (the Western, Eastern and Southern Carpathians), the contribution of the variability between the groups amounted to 9.8% and between the populations within the groups: 20.6% ($F_{ST} = 0.30$). When applying the division of the Carpathian populations based on clustering and ordination results of the Western Carpathian populations vs. Eastern and Southern Carpathian populations, 11.34% of the variability among groups was recorded with $F_{ST} = 0.32$ (Table 2).

Table 2. – Analysis of molecular variance (AMOVA) of *Cicerbita alpina* including different hierarchical levels and geographical groups.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	F_{ST}
1. Among populations	41	2060.01	6.72	30.52	0.30
Within populations	184	2863.37	15.31	69.48	
2. Among geographical groups (Eastern Alps, Western Alps, Eastern Carpathians, Southern Carpathians, Western Carpathians, Balkans, Scandinavia, Sudetes)	7	796.26	2.83	12.57	0.33
Among populations	34	1357.65	4.67	20.69	
Within populations	184	2769.49	15.05	66.74	
3. Among Carpathian groups (Eastern Carpathians, Southern Carpathians, Western Carpathians)	2	269.97	2.18	9.78	0.30
Among populations	24	962.81	4.58	20.61	
Within populations	120	1857.59	15.48	69.61	
4. Among Western Carpathians and Eastern + Southern Carpathians	1	226.28	2.56	11.34	0.32
Among populations	25	1006.49	4.58	20.24	
Within populations	120	1857.59	15.48	68.43	
5. Among Eastern Alps, Western Carpathians & Sudetes	2	123.83	0.62	2.84	0.28
Among populations	20	897.37	5.52	25.07	
Within populations	98	1555.28	15.87	72.09	
6. Among Eastern Alps & Western Alps	1	89.54	2.91	12.96	0.38
Among populations	4	175.84	5.65	25.15	
Within populations	26	361.43	13.90	61.89	
7. Among Western Alps & Balkans	1	121.76	5.44	24.37	0.49
Among populations	4	157.51	5.46	24.46	
Within populations	25	285.70	11.43	51.16	
8. Among Western Carpathians, Eastern Carpathians, Southern Carpathians & Balkans	3	361.76	2.41	10.89	0.31
Among populations	25	977.01	4.51	20.36	
Within populations	126	1916.25	15.21	68.74	
9. Among Scandinavia & Balkans	1	131.56	7.88	33.62	0.51
Among populations	3	93.54	4.09	17.42	
Within populations	20	229.70	11.48	48.96	
10. Among Scandinavia & Western Carpathians	1	128.60	73.74	15.44	0.40
Among populations	16	738.55	5.92	24.40	
Within populations	79	1152.75	14.59	60.16	
11. Among Scandinavia & Eastern Alps	1	89.70	3.94	17.20	0.37
Among populations	3	111.86	4.46	19.44	
Within populations	21	305.43	14.54	63.36	

Discussion

The results of this study do not reveal any strong phylogeographic structure in *Cicerbita alpina*, which implies that the populations forming the present-day range of this species have not been isolated for long time. This inference is congruent with conclusions of a parallel study by Michl et al. (2010) focusing on the western part of this species' range, mainly the Alps. However, certain biogeographical trends emerge from the detailed analyses, which have a few important aspects of historical inference. These trends are discussed below for the Carpathians, which constitute the main part of this work, as well as in a broader geographical context.

Phylogeography of a subalpine herbaceous plant in the Carpathians

The AFLP analysis of Carpathian populations of *Cicerbita alpina* demonstrated the existence of a genetic structure that is correlated with the distribution, with two phylogeographical groups comprising the Western and Southern with Eastern Carpathian populations. These genetic groups are connected with the physical, geographical and phytogeographical divisions, which distinguishes the Western and South-Eastern (Southern and Eastern) Carpathians. This division is based on geological, climatic, habitat and historical differences, whose significance is emphasized in the literature of the 19th century (Herbich 1861, Kotula 1881, Wołoszczak 1896, Pax 1898–1908). The main Carpathian regions are also centres for different groups of endemic species (Pawłowski 1970, Bosçaiu & Täuber 1985). The genetic pattern of *C. alpina* is consistent with the data obtained for the Carpathian populations of several high-mountain species (Mráz et al. 2007, Ronikier et al. 2008a, Thiel-Egenter et al. 2009, reviewed by Ronikier 2011). However, the level of genetic divergence between the groups is lower than in the case of alpine species. *Cicerbita alpina* is a tall plant (up to 2.5 m), producing a large number of light, small diaspores, which can be dispersed over great distances by wind. Occasional dispersion of diaspores over long distances could promote gene flow between geographically distant regions and result in low parameters of genetic isolation. Nevertheless, the genetic structure indicates that there are local, isolated centres of this species' occurrence in different parts of the Carpathians. The Carpathians, unlike the Alps, were not completely covered by glaciers during the Quaternary glaciations. Glaciers covered the highest Carpathian massifs, while the lower massifs and valleys remained un-glaciated (Pawłowski 1970, Mojski 1993, Ronikier 2011). This may have helped alpine and other groups of plants to survive in situ in deep valleys (Pawłowski 1966, 1970). Palynological and paleobotanical data indicate presence of glacial refugia for some tree species in the Carpathian valleys, e.g. *Picea* and *Betula* (Mamakowa & Środoń 1977, Obidowicz et al. 2004, Tanțău 2004, Tanțău et al. 2006). The results of the ordination and clustering (Figs 4B, 5) confirm the bio-geographical significance of the barrier between the Western and Eastern Carpathians, proved previously for alpine species (cf. Mráz et al. 2007, Ronikier 2011) and for species occurring much lower down in forests and humid valleys, thus not fully supporting our hypothesis of extensive glacial gene exchange among tall-herb plant populations at the scale of the Carpathians. We suggest that the extant Western Carpathian populations originated from different refugia than the Eastern and Southern Carpathian populations. Taking into account the range of the last glaciation, which had a major influence on the Western Carpathians (Lindner 1988, Mojski 1993), and low values of genetic indices and different

genetic patterns for *Cicerbita alpina*, refugia for the Western Carpathians should be searched for in deep valleys in the southern part of the range. Populations in the Eastern and Southern Carpathians are characterized by high similarity, both at the level of populations and individuals. This could be indirect evidence for the common origin of populations of these geographical regions and the possibility of a relatively constant exchange of genetic material. There is no clear geographical barrier between the Southern and Eastern Carpathians. Clear genetic differences in the case of alpine species *Campanula alpina* and subalpine *Hypochoeris uniflora* indicate there were different refugia for these regions (Mráz et al. 2007, Ronikier et al. 2008a). It seems that the genetic similarity of *Cicerbita alpina* in this region results from higher tolerance of this species of unstable climatic conditions and greater possibility of vertical migration. Also the biological characteristics could be significant, such as the adaptation for dispersal by wind. The influence of biological properties, including mainly pollination and dispersal, was also invoked to explain the lack of genetic structure in anemophilous *Carex curvula* within the South-Eastern Carpathians (Puşcaş et al. 2008). In this context, however, the populations from the Bucegi and Făgăraşului Mts are characterized by surprisingly high genetic indices: e.g. DW (8.23 and 6.8, respectively) and percentage of polymorphism (37.7% and 43.2%, respectively), which may suggest the existence of local refugia in this area, which were important in determining the high level of genetic diversity.

The differences between the genetic structure of the Western and Southern plus Eastern Carpathian populations are reflected not only in the presence of separate groups, but also in the degree of internal heterogeneity within the groups (Figs 2, 3). The individuals from the Southern and Eastern Carpathians form an almost homogeneous cluster in the Bayesian analysis, whereas the genetic structure of those from the Western Carpathians is much more complex, which may indicate a greater isolation of local populations and/or possibility of secondary contacts. There is a group of populations within the Western Carpathians that have an island-like geographical position at the periphery of the area (PM, PN). They are characterized by low genetic indices and formed subgroups in the numerical analyses (Table 1, Fig. 5). Stachurska-Swakoń et al. (2011) suggest that such populations could represent traces of persistent in situ micro-refuges, although the pattern could also result from founder effects and genetic drift.

The Carpathians in the context of other distribution areas: the general phylogeographical pattern

The broader scale genetic structure of *Cicerbita alpina* does not reveal any clearly distinguishable genetic groups that would indicate the presence of different lineages resulting from a long period isolation of regional gene pools. The PCoA analysis, however, indicates two main genetic groups, which could be connected with historical factors. Further subgroups are distinguished by the STRUCTURE clustering and the NJ tree (Figs 2, 3, 4A). This main genetic division is apparent along the NW–SE geographical axis and runs through the Carpathians, dividing the Western and South-Eastern Carpathians not only in terms of the regional data set but also in the general pattern. This further supports the independent history of the two main parts of the Carpathians, as well as the significance of the intra-Carpathian boundary for the biogeography of the mountain flora in Central Europe (Fig. 3). This pattern in the variation implies the existence of two main genetic lines in the

history of the present-day range: a Central-European-northern and south-eastern one. While over most of the range, these lines are allopatric, both could have been important for the extant range in the Alps. A consistent genetic group is formed by populations that occur on the isolated massifs of Central Europe – the Western Carpathians, Sudetes and Eastern Alps. This group includes most of the populations studied from the central part of the range. Interestingly, the Sudetes show a somewhat transitional position within the group (see Figs 2, 4A), i.e. populations from the Western Sudetes seem to be more closely related to those in the Eastern Alps and those in the Eastern Sudetes to those in the Western Carpathians. This may indicate that the modern flora of the Sudetes developed as a result of migrations from different refugia. The transitional position of these populations is also supported by high genetic diversity indices. At present, the flora of the Sudetes, particularly the subalpine and alpine species, is more impoverished than that of the Western Carpathians (Pawłowski 1969). It is believed that this floristic poverty results from a more disastrous influence of glaciations (Pawłowski 1969, Starkel 1991). On the other hand, the presence of Arctic species in the poor alpine flora of the Sudetes, which are considered to be glacial relics (e.g. *Rubus chamaemorus*, *Salix lapponum*, *Saxifraga nivalis*), could imply a closer contact with the periglacial flora of the tundra. The affinity of present-day mountain populations of *C. alpina* with the Western Carpathians confirms previous observations indicating that the Sudetes are not significantly genetically distinct, while previous studies indicate a clearer connection with the Carpathians than the Alps (Fér et al. 2007, Mitka et al. 2007, Ronikier et al. 2008a, Ronikier & Szelağ 2008, Těšitel et al. 2009, reviewed by Ronikier 2011). Interactions between these two floras in the past are also revealed by the presence of Western Carpathian subendemics in the Sudetes (Pawłowski 1969, Fabiszewski 1970). A common origin of both the Carpathian and Sudetic genetic stock of *Aconitum* sect. *Aconitum* or secondary contact(s) between the two mountain ranges are also postulated by Mitka et al. (2007) based on cytogenetic and ISSR data. On the other hand, species may have also migrated to the Sudetes from the West through the central German mountains (Pawłowski 1966), which would explain the affinity of Western Sudetes individuals with Eastern Alpine populations.

Populations from Scandinavia show affinity with Central European populations and we suggest that populations from this area were the source of the postglacial colonization of Scandinavia. Even though the Bayesian analysis separated the Scandinavian individuals, PCoA confirms their similarity. AMOVA revealed little differentiation between the Scandinavian populations and those from the Eastern Alps and Western Carpathians (Figs 2, 4A; Table 2) and values of genetic similarities between populations (Nei's Original Measures of Genetic Identity) confirm this similarity. The genetic structure of *C. alpina* constitutes yet further evidence of the significance of Central Europe for the development of the present-day flora of Scandinavia (reviewed by Ronikier 2011). Phylogeographic studies and paleobotanical data imply that the present-day diversity of the Scandinavian flora originated from both postglacial re-colonization, including long-distance colonization and processes of speciation such as hybridization or polyploidization (Brochmann & Brysting 2008). As already suggested by Michl et al. (2010), periglacial populations of *C. alpina* could have existed during the last glaciation in mountains north of the Carpathians and Alps and have been important in the stepwise colonization of Scandinavia. The potential significance of the so-called northern refugia for several mountain species of different ecological characters, e.g. *Arabis alpina*, *Pulsatilla vernalis* and *Saxifraga oppositifolia*,

is discussed in the literature (Holderegger et al. 2002, Ehrich et al. 2007, Ronikier et al. 2008b). Paleobotanical data indicate the existence of cryptic refugia in Central and eastern Europe during the LGM, within which woody and alpine species persisted (Ralska-Jasiewiczowa et al. 2004, Bhagwat & Willis 2008, Birks & Willis 2008). However, it is also likely that long distance dispersal from the mountains was the basis of the postglacial colonization of Scandinavian by *C. alpina* as the plants there are genetically similar to those in extant Central European populations.

Our results indicate the populations studied from the Southern and Eastern Carpathians and Balkans are genetically similar. The latter are not very polymorphic but have the highest number of characteristic bands and a relatively high DW index. One of the populations from the Southern Carpathians (RT, Retezat Mts) is similar to the Balkan populations (Nei's Original Measures of Genetic Identity: RT–BH = 0.93, RT–BR = 0.93), which may imply a relatively recent gene exchange. During glacial periods montane forests descended below an altitude of 1000 m a.s.l. in the Bulgarian Rila Mts (Bozilova & Tonkov 2000). Low levels of genetic divergence may result from frequent secondary contact at low altitudes during glacial periods. In turn, there is a genetic division within the Alpine populations into the Western and Eastern Alps. We suggest that *C. alpina* could have survived the last glaciation in local, peripheral refugia, like some alpine species (Schönswetter et al. 2005). This is also in general line with the results of Michl et al. (2007, 2010) who suggest a different origin of the contemporary populations of *C. alpina* in the Southern Alps and the rest of its Alpine distribution. A division into phylogeographic groups restricted to the Eastern and Western Alps is also recorded for *Picea abies* (Scotti et al. 2000). Since currently *C. alpina* occurs in spruce forests in the subalpine zone, both species may have had common refugia, which were different for different parts of the Alps.

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Souhrn

Práce se zabývá fylogeografií *Cicerbita alpina*, typického druhu subalpínských společenstev evropských pohoří. Byly analyzovány rostliny z celého areálu druhu, především však z Karpat, které jsou významným pohořím z hlediska fyto geografie horské flóry Evropy. Analýza metodou AFLP neodhalila výraznou fylogeografickou strukturu, což naznačuje, že populace z jednotlivých částí současného areálu se nevyvíjely v dlouhodobé izolaci, ale pravděpodobně zůstávaly v příležitostném kontaktu v důsledku dobré schopnosti šířit se na velkou vzdálenost. Genetická struktura nicméně ukazuje na určité fylogeografické trendy, které odrážejí přežívání v lokálních refugiiích a následnou diverzifikaci samostatných vývojových linií během posledního zalednění. V rámci Karpat je patrné rozdělení populací na západokarpatské a jihovýchodokarpatské, z nichž první jsou blízké příbuzné rostlinám z východních Alp a Sudet a druhé jsou podobné balkánským populacím. Skandinávské populace mají po genetické stránce blízko k západokarpatským a vznikly ve východních Alpách nebo západních Karpatech; na tomto vývoji se patrně jako mezistupěň podílely populace ze severněji položeného refugia.

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