Genetic Differentiation among Populations of *Cicerbita alpina* (L.) Wallroth (Asteraceae) in the Western Alps

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In this study we analyzed the genetic population structure of the hygrophilous tall-herb *Cicerbita alpina* in the western Alps because this group of mountain plants is underrepresented in the biogeographical literature. AFLP (amplified fragment length polymorphism) fingerprints of 40 samples were analyzed from four populations situated in a transect from the southwestern Alps to the eastern part of the western Alps and one population from the Black Forest outside the Alps. Two genetic groups can be distinguished. The first group (A) comprises the populations from the northern and eastern parts of the western Alps, and the second group (B) comprises the populations from the southwestern Alps and the Black Forest. Group A originates most likely from at least one refugium in the southern piedmont regions of the Alps. This result provides molecular evidence for a humid climate at the southern margin of the Alps during the Würm glaciation. Group B originates presumably from western or northern direction and we discuss two possible scenarios for the colonization of the Alps, \textit{i.e.} (1) long-distance dispersal from southwestern refugia and (2) colonization from nearby refugia in the western and/or northern Alpine forelands. The study demonstrates that the target species harbours considerable genetic diversity, even on a regional scale, and therefore is a suitable model for phylogeographic research.

\textit{Key words: AFLP, Cicerbita, Molecular Phylogeography}

\section*{Introduction}

The Pleistocene landscape during the Last Glaciation Maximum (LGM, 13,000–18,000 a before present) is described on the basis of pollen maps and palaeoclimatic reconstruction as continuous tundra and cold steppe on permafrost (Lang, 1994). This assumption has been supported by several molecular investigations into the biogeographic history of plants and animals demonstrating that many taxa actually occurring in the temperate zone had refugia in southern Europe during the cold stages (Hewitt, 2004). In addition, recent molecular and fossil studies suggest that microclimatically buffered regions within the periglacial existed, where even thermophilous and/or hygrophilous taxa persisted the cold stages of the ice ages (\textit{e.g.} Willis \textit{et al.}, 2000; Stewart and Lister, 2001).

However, phylogeographic surveys concerning Central Europe and the Alps have thus far emphasized animals (\textit{e.g.} \textit{Arion fucus}, Pinceel \textit{et al.}, 2005; \textit{Erebia epiphron}, Schmitt \textit{et al.}, 2006), trees (Petit \textit{et al.}, 2003), herbaceous plants of Alpine meadows and screes (\textit{e.g.} \textit{Pritzelago alpina}, Kropf \textit{et al.}, 2003; further taxa see Stehlík, 2003 and Schönswetter \textit{et al.}, 2005) and submediterranean-temperate mountain elements (\textit{Anthyllis montana}, Kropf \textit{et al.}, 2002; \textit{Meum athamanticum}, Huck, unpubl.). Plants that depend on cool and humid climates and are sensitive to strong wind are underrepresented, even though such elements should be indicators for humid regions or microclimatically buffered sites in the periglacial. Only Despres \textit{et al.} (2002) analyzed the montane-subalpine \textit{Trollius europaeus} in a widespread phylogeography, but with a low sampling density in Central Europe.

Further studies on species that actually occur in humid mountain habitats may provide invaluable additions to our understanding of vegetation history, for example the montane-subalpine broad-leaved tall-herb *Cicerbita alpina*. It exhibits a suitable distribution pattern for phylogeographic analyses. This species occurs in almost all mountain
ranges of Europe. Furthermore, if suitable habitats are abundant, the species populations are mostly composed of many individuals.

The western Alps (W Alps) were chosen as a study area since data on several other taxa from this region are available that can be compared to our results. Furthermore, the available molecular studies outline the W Alps as an interesting region to test biogeographic hypotheses. Here, the ice cap of the Würm glaciation almost reached the Mediterranean sea during the LGM and formed an effective barrier not only for northern and southern, but also for western and eastern populations. After deglaciation many species colonized this area from several isolated refugia (Hewitt, 2004). Consequently, formerly isolated lineages came into secondary contact, leading to hybridization and speciation (e.g. Kropf et al., 2002). Several contact zones have been found in surveys of intraspecific differentiation conducted on plant and animal taxa. Studies on temperate, albeit cold-adapted species detected colonization by lineages that originated north and south of the Alps (Clethrionomys glareolus, Deffontaine et al., 2005; Melum athamanticum, Huck, unpubl.), whereas Kropf et al. (2002) detected lineages of the submediterranean Anthyllis montana that originated in western and southeastern Europe.

For the central parts of the W Alps, we propose a hypothesis of total extinction of Cicerbita alpina during the LGM, because the species occurs today exclusively in montane-subalpine tall-herb, scrub and forest vegetation and is not adapted to the high alpine climate and the strong winds that are assumed for ice-free mountain tops within the continuous ice sheet ('nunataks', see e.g. Stehlík, 2003). In the piedmont regions of the Alps glacial persistence can be expected along streams of melted snow and ice. Actually, the species is often found in humid habitats near glaciers (T. Michl, unpubl.). A relatively humid climate during the LGM is proposed, especially for the southern margin of the Alps (Florineth, 1998). Boșcaiuc and Tăuber (1980) hypothesized glacial persistence of subalpine scrubs (Pinus mugo, Alnus incana) and associated tall-herbs also in periglacial areas in protected microhabitats.

To confirm these different hypotheses we aimed to compare five populations of C. alpina using AFLPs (amplified fragment length polymorphisms). In this article we address the following questions: (1) Does a genetic population structure exist among populations of Cicerbita alpina? (2) What hypotheses and conclusions concerning glacial refugia can be stated?

Materials and Methods

Plant material and sampling

In summer 2004 we sampled leaves from five populations of Cicerbita alpina (Fig. 1, Table I). The sampling of the populations was arranged in transects from the southwestern to the eastern part of the W Alps and to the north including one population from the former periglacial area. The collectors of the material were: D. Eisenacher (PE) and T. Michl (other populations). The population size was estimated and was in all populations > 1,000 individuals. Leaf material was collected in situ across the five populations from at least 12 different plants and dried in plastic zip-lock bags filled with silica gel. To avoid sampling of clones, the material was collected at distances of at least 10 m. Finally, eight individuals per population were included in the AFLP analysis.

DNA extraction and AFLP fingerprinting

Genomic DNA was extracted from silica gel-dried leaves using the ‘DNeasy Plant Mini Kit’

Table I. Population codes (alphabetically), sample locations, mountain ranges, countries, coordinates, altitude, number of individuals per population (N), percentage of polymorphic loci, and Shannon’s gene diversity index for sampled populations of Cicerbita alpina.

<table>
<thead>
<tr>
<th>Pop. code</th>
<th>Location</th>
<th>Mountain range</th>
<th>Country</th>
<th>Coordinates</th>
<th>Altitude [m a. s. l.]</th>
<th>N</th>
<th>Polymorphic loci (%)</th>
<th>Shannon’s index</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>Col du Lautaret</td>
<td>SW Alps</td>
<td>France</td>
<td>06°25′E 45°00′N</td>
<td>2050</td>
<td>8</td>
<td>21.64</td>
<td>0.1188</td>
</tr>
<tr>
<td>FB</td>
<td>Feldberg</td>
<td>Black Forest</td>
<td>Germany</td>
<td>08°00′E 47°55′N</td>
<td>1300</td>
<td>8</td>
<td>47.01</td>
<td>0.2325</td>
</tr>
<tr>
<td>FE</td>
<td>Ferret</td>
<td>W Alps</td>
<td>France</td>
<td>07°08′E 45°55′N</td>
<td>1500</td>
<td>8</td>
<td>64.93</td>
<td>0.3417</td>
</tr>
<tr>
<td>OB</td>
<td>Oberalppass</td>
<td>W Alps</td>
<td>Switzerland</td>
<td>08°40′E 46°40′N</td>
<td>2000</td>
<td>8</td>
<td>49.25</td>
<td>0.2838</td>
</tr>
<tr>
<td>PE</td>
<td>Refuge Pernici</td>
<td>eastern W Alps</td>
<td>Italy</td>
<td>10°45′E 45°55′N</td>
<td>1600</td>
<td>8</td>
<td>50.75</td>
<td>0.2879</td>
</tr>
</tbody>
</table>
Fig. 1. Collection localities with genetic variation based on Shannon’s diversity index ($H_{SH}$, left symbol) and number of private fragments ($f_{pr}$, $f_{prf}$, right symbol). Medium black line (partly dashed), shape of Alpine massif; grey colour, ice sheet of the Alps during the LGM (based on Florineth and Schlüchter, 1998, slightly modified). Semicircles: extra large, $H_{SH} \geq 0.21$ resp. $f_{pr} + f_{prf} \geq 6$; large, $0.19 \leq H_{SH} < 0.21$ resp. $f_{pr} + f_{prf} = 4$ and 5; medium, $0.17 \leq H_{SH} < 0.19$ resp. $f_{pr} + f_{prf} = 2$ and 3; small, $0.15 \leq H_{SH} < 0.17$ resp. $f_{pr} + f_{prf} = 1$; small open, $H_{SH} < 0.15$ resp. $f_{pr} + f_{prf} = 0$. For abbreviations and details see Tables I and II.

(Afgen, Hilden, Germany) according to the manufacturer’s instructions.

AFLP procedures followed Vos et al. (1995) with minor modifications as follows. Purified DNA was eluted in 50 μL instead of 100 μL. The analysis was made with fluorescence-labelled primers [6-FAM, NED, HEX, Applied Biosystems (ABI), Norwalk, Connecticut, USA] instead of radioactive labelled. For each sample, genomic DNA concentration was determined (Alpha Imager, Alpha Innotech, San Leandro, USA) and standardized to 20 ng DNA/μL. For digestion 100 ng genomic DNA were used. The initial restriction-ligation step was performed for 14 h at 23 °C. Genomic DNA was digested simultaneously with EcoRI and MseI. Double-stranded EcoRI and MseI adapters were ligated to the sticky ends of the fragments. In the following two-step amplification, preselective primers with one selective base (EcoRI primer E +1, MseI primer M +1) and selective primers with three additional selective bases (EcoRI primer E +3, MseI primer M +3) were used. The analysis was performed using three primer combinations, i.e. E37/M54, E39/M61, E45/M57. The primer sequences used were: E, 5’-GACTGCGTACCAATTCA-3’; M, 5’-GATGAGTCCGAGTAGGAC-3’; E37/M54, E + ACG/M + CCT; E39/M61, E + AGA/M + CTG; E45/M57, E + ATG/M + CGG.
Multiplex products were run for 4 h on an ABI 377 sequencer to separate fragments together with an internal size standard (GeneScan ROX 500, ABI). Fragments were automatically scored with the GENOTYPER analysis software (version 2.1, ABI). Fragments were scored as present when the appropriate peak height exceeded the standard parameter-setting thresholds (for blue, 60; green, 30; yellow, 40; red, 40). In the finally assembled binary matrix the presence of a band was scored as 1, whereas the absence of the band was scored as 0. In order to check for possible misinterpretations of the automated GENOTYPER analysis all electropherograms were reexamined visually.

Data analysis

Estimates of genetic distances \((D)\) among individuals were calculated from the binary matrix using a complementary value \((SC)\) based on Nei and Li’s (1979) similarity coefficient \(\hat{S}\) \((D = 1 - SC = 1 - [2n_{xy}/(n_x + n_y)]\); see Kropf et al., 2002). Based on the resulting distance matrix, relationships between all individuals were reconstructed using neighbour-joining (NJ) analysis (Saitou and Nei, 1987) as implemented in PAUP version 4.0b10 (Swofford, 1998). The resulting neighbour-joining phenogram (Fig. 2) was calculated using Nei’s (1978) unbiased genetic distances between all pairwise combinations of samples. Node support of the phenogram was estimated by a bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

Additionally, the Bayesian multilocus assignment method of Corander et al. (2003) as implemented in the software BAPS (version 3.2) was used to infer the genetic structure of the dataset. The program requires an \textit{a priori} expectation for the maximum number of groups \((K)\) and it allows multiple inputs of \(K\). Values of \(K\) ranging from 1 to 5 (the number of studied populations) were explored, using 20 replicates each (Corander et al., 2003).

Furthermore, principal co-ordinates (PCOs) and minimum spanning tree (MST) analyses were performed to illustrate overall similarity among individuals using NTSYS-PC version 2.1 (Rohlf, 2000). PCOs were extracted from the squared Euclidean distances between all pairs of AFLP phenotypes (Gower, 1966; Sneath and Sokal, 1973). The PCO was conducted using the programs SIMINT, DCENTRE, EIGEN and MATRIX of NTSYS-PC. The program MST of NTSYS-PC was used to calculate the MST. Finally, the MST was superimposed onto the PCO plot to show similarities among individuals when all dimensions were taken into account.

Differentiation between populations was described using \(F\) statistics (Wright, 1931, 1951) derived within the framework of the analysis of molecular variance (AMOVA, Excoffier et al., 1992) as implemented in ARLEQUIN v2.0 (Schneider et al., 2000). ARLEQUIN was also used to generate a matrix of pairwise \(F_{ST}\) values among the five populations (Weir and Cockerham, 1984). AMOVAs were carried out for the entire dataset and the two geographical subsets inferred in the cluster analyses in order to compare levels of among-population differentiation between geographic groups. Significance levels of variance components were computed by a nonparametric permutation approach with 1000 replicates.

In order to compare levels of genetic diversity within populations, three different diversity estimators were calculated: (1) Shannon’s diversity index \(H_{SH}\) with \(H_{SH} = -\sum (p_i \ln [p_i])\), where \(p_i\) is the relative frequency of the \(i\)-th fragment (Legendre and Legendre, 1998); (2) percentage of polymorphic loci of all polymorphic loci at the 99% level \((\%P_{pop})\); and (3) Nei’s gene diversity \(H\) (Nei, 1973). Calculation of \(\%P_{pop}\) and \(H\) is implemented in POPGENE (Yeh et al., 1997). The number of private, rare and sparse fragments per population and group of populations were extracted from the binary matrix. Private fragments are confined to a single population or group. Fragments were treated as rare when they occurred in less than 25%, and treated as sparse when they occurred in more than 25% and less than 50% of the 40 investigated individuals.

Results

AFLPs

The three selective AFLP primer combinations amplified a total of 134 fragments. The primer combination E37/M54 yielded 40 fragments ranging from 132 to 495 base pairs in size. E39/M61 yielded 78 fragments ranging from 135 to 485 base pairs in size, and E45/M57 yielded 19 fragments ranging from 174 to 230 base pairs in size. In total 90.6% of the fragments were polymorphic, 9.4% were scored in all individuals from all populations and no identical AFLP profiles were found.
Fig. 2. Neighbour-joining (NJ) phenogram of 40 individuals from five populations of *Cicerbita alpina* based on genetic distances (based on Nei and Li, 1979). Groups A and B are the phylogeographic groups described in the text. For abbreviations and details see Table I.

**Genetic relationships among populations**

In the neighbour-joining phenogram (Fig. 2) all individuals sampled from the same population grouped together, except for two outlying individuals from population Oberalppass (OB). Two main clusters of populations can be distinguished. One cluster contains all individuals from Ferret (FE) and Oberalppass in the W Alps, and Refuge Pernici (PE) from the eastern part of the W Alps, referred to as group A. The other cluster contains the population from Col du Lautaret (CL) in the southern W Alps and the population from the Black Forest (FB), referred to as group B. However, none of these groupings is supported by bootstrap values > 70%.

More detailed genetic structure is revealed by the model-based Bayesian clustering analysis (not shown). Highest posterior probabilities (0.6273) were obtained for $K = 5$. Populations CL and FB form one group. A second cluster is composed of the population PE. With the exception of one individual, population OB forms a third cluster. Another cluster is composed of individuals from population FE. Finally the program separated two individuals from population FE into one separate cluster.

The results of the PCO analysis (Fig. 3) largely confirmed the results of the NJ and BAPS analyses. The two groups (A and B) are separated along the first axis of the PCO, which explains 22.5% of the overall variation. The substructure found in the PCO analysis confirms a higher differentiation within group A than in group B.

Fig. 3. Principal co-ordinates (PCOs) and minimum spanning tree (MST) analyses of all individuals of *Cicerbita alpina* based on their squared Euclidean distances among AFLP phenotypes. Ordination axis 1 vs. 2 is shown. These two axes explain 22.5% and 8.9% of the overall variation, respectively. Black triangles, OB; black squares, FE; black dots, PE; open squares, FB; open circles, CL.
The NJ and the PCO detected one transitional individual each. The NJ detected one transitional individual from FB (group B) to population OB (group A) and the PCO detected one transitional individual from population OB to population FB.

Regarding $F_{\text{ST}}$ values, all ten pairwise genetic distances between populations were highly significant ($p < 0.001$). Average $F_{\text{ST}}$ was 0.3530. Maximum $F_{\text{ST}}$ was 0.5509, observed between population OB from the W Alps and the population CL from the southern W Alps, followed by 0.4477 between FE from the W Alps and CL. Minimum $F_{\text{ST}}$ was 0.1847, found between population FB from the Black Forest and population CL, followed by 0.2413 between the W Alpine populations FE and OB.

**Populational and regional levels of genetic diversity**

The five examined populations of *Cicerbita alpina* showed varying levels of intrapopulational diversity (Table I, Fig. 1). Average diversity values in all populations were: $\%P_{\text{pop}}$, 46.72%; $H_{\text{SH}}$, 0.253; $H$, 0.171. The highest diversity was found in population FE ($\%P_{\text{pop}}$, 64.93%; $H_{\text{SH}}$, 0.342; $H$, 0.228) from the W Alps followed by PE ($\%P_{\text{pop}}$, 50.75%; $H_{\text{SH}}$, 0.288; $H$, 0.197) and OB ($\%P_{\text{pop}}$, 49.25%; $H_{\text{SH}}$, 0.284; $H$, 0.195). The lowest genetic diversity was found in population CL ($\%P_{\text{pop}}$, 21.64%; $H_{\text{SH}}$, 0.119; $H$, 0.081). The three different indices are significantly correlated [pairwise Spearman's rank correlation coefficient ($r_s$), $\%P_{\text{pop}}$ vs. $H_{\text{SH}}$, 0.97; $H_{\text{SH}}$ vs. $H$, 0.94; $\%P_{\text{pop}}$ vs. $H$, 0.93; all $p < 0.001$].

Every population exhibited private fragments (Table II). The highest number of private fragments was found in population FE (14) whereas population CL exhibited only one. Regarding rare fragments, populations FE (24), followed by OB (19) and FB (12) showed the highest numbers, whereas population CL exhibited only one rare fragment. Furthermore, the populations showed comparable proportions of sparse fragments.

### Table II. Private, rare and sparse fragments per population and group of populations.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pop.</th>
<th>Private fragments</th>
<th>Rare fragments</th>
<th>Sparse fragments</th>
<th>Fragments (&gt;50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>in pop. in group</td>
<td>(&lt;25%) in pop. in group</td>
<td>(25–50%) in pop. in group</td>
<td>in pop.</td>
</tr>
<tr>
<td>A</td>
<td>FE</td>
<td>14 34</td>
<td>24 48</td>
<td>37 40</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>OB</td>
<td>3 19</td>
<td>39</td>
<td>32</td>
<td>41</td>
</tr>
<tr>
<td>B</td>
<td>CL</td>
<td>1 0</td>
<td>19</td>
<td>9 22</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td>5 12</td>
<td>18</td>
<td>18</td>
<td>10</td>
</tr>
</tbody>
</table>

### Table III. Analysis of molecular variance (AMOVA) of AFLP phenotypes. The total data set contained 40 individuals from five populations. The two geographical groups (A and B) are based on the results of the NJ analyses (Fig. 2). Degrees of freedom ($df$), sum of squares ($SSD$), variance component estimates ($CV$), percentage of total variance (%total), and significance value for among-population variation ($P$) are given.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>$df$</th>
<th>$SSD$</th>
<th>$CV$</th>
<th>%total</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among all populations</td>
<td>4</td>
<td>287,700</td>
<td>7.36920</td>
<td>36.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>35</td>
<td>454,000</td>
<td>12.97143</td>
<td>63.77</td>
<td></td>
</tr>
<tr>
<td>Two groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>1</td>
<td>129,179</td>
<td>3.97598</td>
<td>18.13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>3</td>
<td>158,521</td>
<td>4.98361</td>
<td>22.72</td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>35</td>
<td>454,000</td>
<td>12.97143</td>
<td>59.15</td>
<td></td>
</tr>
<tr>
<td>Group A (FE–OB–PE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among all populations</td>
<td>2</td>
<td>133,083</td>
<td>6.36905</td>
<td>29.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>21</td>
<td>327,375</td>
<td>15.58929</td>
<td>70.59</td>
<td></td>
</tr>
<tr>
<td>Group B (FB–CL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among all populations</td>
<td>1</td>
<td>25,438</td>
<td>2.04911</td>
<td>18.47</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>14</td>
<td>126,625</td>
<td>9.04464</td>
<td>81.53</td>
<td></td>
</tr>
</tbody>
</table>
On a regional scale, 34 private fragments were found in group A, whereas in group B none was found (Table II). 19 of the 34 private fragments of group A are shared by all three populations, 12 are shared by the FE and OB population whereas two are shared by the FE and PE population and one is shared by the OB and PE population.

The non hierarchical AMOVA assigned 36.2% of the overall genetic variation to variation among populations (Table III). In the groupwise hierarchical AMOVA group A exhibited 29.0% among populations, whereas group B exhibited 18.5% of variation among populations.

The stronger differentiation among the populations of group A is in line with results of the NJ, PCO and BAPS analyses. The NJ exhibits longer internal branches within this group in general, the PCO separates these populations to a higher extent and BAPS assigns populations of this group to distinct clusters.

**Discussion**

**Genetic lineages of Cicerbita alpina in the Western Alps**

The W Alps are colonized by *Cicerbita alpina* from at least two directions. The results suggest one lineage from western or northwestern direction and one lineage from southeastern direction. This is supported by the NJ and PCO analyses (Figs. 2 and 3) and the pairwise $F_{ST}$ values (see results). All three reveal the largest genetic distinction between the corresponding groups A (FE, OB, PE) and B (CL, FB). Only two transitional individuals from populations OB and FB, respectively (Figs. 2 and 3), indicate a weak gene flow between these two groups.

In addition to the genetic differentiation between the two deduced lineages, the populations of group A show a remarkable higher genetic diversity (Table I, Fig. 1) and in the PCO analysis group A is more scattered (Fig. 3). There is a remarkably high number of private fragments within this group (Table II). Additionally, the populations of group A share a high number of private fragments, supporting their definition as a discrete lineage. In contrast to the populations of group A, the populations of group B show considerable lower genetic diversity (Table I, Fig. 1) and fewer private fragments (Table II).

Concerning the genetic differentiation of the two lineages, a comparable pattern was shown for the Mediterranean-montane *Anthyllis montana* by Kropf et al. (2002). The authors detected a contact zone of a western (Cantabric Mountains, Pyrenees, Cevennes and French Alps) and eastern lineage (Italian Mediterranean Alps, eastern Alps and Balkan peninsula) on Col de Tende in the Mediterranean Alps. Flanagan et al. (1999) also detected hybrid zones of a widespread grasshopper (*Chorthippus parallelus*) on two passes (Col Larche, Passo de Resia) in the western and Central Alps. They found hybridization of the lineages that populate Central Europe north of the Alps and the Italian peninsula south of the Alps. *Cicerbita alpina* and *Chorthippus parallelus* have a comparable upper distribution limit of approximately 2,000 m a. s. l. A similar contact zone roughly situated along the main Alpine watershed seems possible, a dispersal of *C. alpina* may be limited due to the expansion of the alpine and the snow belt and the cliffy topography of the Alps.

**Scenario for the southeastern lineage (group A)**

The high values of the diversity indices and the high numbers of private fragments of group A most likely reflect the proximity of a glacial refugium. This is reasonable because the greater age of refugial populations enables them to accumulate more private fragments (assuming they did not undergo severe bottlenecking). During small-scale migration from the refugia to the sampled localities much of the remnant genetic diversity and private fragments are conserved due to a slow and steady stepping stone dispersal (Hewitt, 1996). We therefore conclude that current-time populations of group A originate from refugia situated in the southern piedmont regions of the Alps. These regions have been free of inland ice and are often considered to be refugia, especially for alpine taxa (e.g. Hewitt, 2004; Schönswetter et al., 2005), but also for the thermophilous *Anthyllis montana* (Kropf et al., 2002) and for the temperate forest mammal *Clethrionomys glareolus* (Deffontaine et al., 2005). It is also proposed that the climate at the southern margin of the Alps during the LGM was not as dry as in the northern and eastern forelands of the Alps (Florineth, 1998). Furthermore, Tribsch and Schönswetter (2003) deduced on the basis of palaeo-environmental data of the southeastern Alps that the treeline was around 800 m a. s. l. and subalpine conditions were present up to 1,200 m a. s. l. during the LGM. The results con-
cerning Cicerbita alpina confirm the assumptions of Tribsch and Schönswetter (2003) about the glacial persistence of boreal woodland in the southern piedmont regions because this vegetation type is associated with tall-herbs today.

Remarkable is the considerably higher genetic diversity and higher number of private fragments in the FE population (Table II) and the eastward decrease in the number of shared private fragments within group A (see Results). This genetic structure could have been formed by dispersal from one refugium that was situated near the FE population. But the lower indices of populations OB and PE may also simply be due to smaller refugial populations in separate refugia. Indeed, the private fragments of the latter populations more likely indicate that the southern piedmont regions of the Alps have been a substructured refugial area for Cicerbita alpina during the LGM.

**Two scenarios for the northwestern lineage (group B)**

The low values of the diversity indices and the low numbers of private fragments of group B may be due to bottlenecks in small refugial populations or due to founder effects after colonization from distant refugia.

Regarding the first possibility, small refugial populations could have existed in the periglacial west and north of the Alps. The lower genetic diversity and lower number of private fragments of the CL population in contrast to the FB population (Tables I and II) indicate that the southern W Alps are more likely colonized from a refugium north of the W Alps, e.g. in the surroundings of the Black Forest. Northern refugia have also been detected for plant species that today occur up to boreal regions (e.g. Calluna vulgaris, Rendell and Ennos, 2002) and for a temperate plant (Meum athamanticum, Huck, unpubl.). Several studies on animals also found northern lineages that give hints to cryptic northern refugia (e.g. Schmitt and Seitz, 2001; Deffontaine et al., 2005; Pinceel et al., 2005). However, also in the western forelands of the Alps, periglacial refugia have been proposed for the butterfly Erebia epiphron (Schmitt et al., 2006). Like Cicerbita alpina, this butterfly requires a humid climate (Schmitt et al., 2006).

Secondly, it is also plausible to assume a distant southwestern refugium, i.e. the Iberian peninsula as a source for the colonization of the southern W Alps and the Black Forest. The lower genetic diversity of the CL population can be explained by a more recent colonization event. This scenario is in accordance with results for many plant and animal taxa, which point to the Iberian peninsula as one of the major ice age refugia in Europe for species not adapted to an arctic climate (Hewitt, 1999; Petit et al., 2003). For several other taxa exchange of migrants between the Pyrenees and W Alps was also assumed (e.g. Phyteuma globularifolium, Schönswetter et al., 2002; Trollius europaeus, Despres et al., 2002). Schönswetter et al. (2002) proposed dispersal of P. globularifolium via the French Massif Central, which acted temporarily as a stepping stone through the time when this region exhibited an alpine-belt climate. This can also be assumed for Cicerbita alpina, which currently appears in the Massif Central and in addition to P. globularifolium has a propensity for long-distance dispersal, aided in the case of C. alpina by a plume-like parachuting structure on the seed.

The genetic structure of the analyzed hygrophyllous tall-herb Cicerbita alpina is congruent with patterns in the among-population structure of many plant species of other mountain habitats and also with the pattern of several animal species. The rugged topography of the Alps contributes to a high interpopulational genetic variation and the maintenance of different lineages. Therefore, our study documents the explanatory genetic variation for phylogeographic analyses.

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