Historical divergence vs. contemporary gene flow: evolutionary history of the calcicole \textit{Ranunculus alpestris} group (Ranunculaceae) in the European Alps and the Carpathians

O. PAUN,\textsuperscript{*}† P. SCHÖNSWETTER,‡ M. WINKLER,§ INTRABIODIV CONSORTIUM¶ and A. TRIBSCH**

\textsuperscript{*}Molecular Systematics Section, Jodrell Laboratory, Royal Botanic Gardens, Kew, Surrey TW9 3DS, UK, †Department of Systematic and Evolutionary Botany, ‡Department of Biogeography and Botanical Garden, University of Vienna, Rennweg 14, A-1030 Vienna, Austria, §Department of Integrative Biology, University of Natural Resources and Applied Life Sciences, Gregor-Mendel-Str. 33, A-1180 Vienna, Austria, ¶http://www.intrabiodiv.eu/IMG/pdf/IntraBioDiv_Consortium.pdf, **Department of Organismic Biology/Ecology and Diversity of Plants, University of Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria

Abstract

Although many species have similar total distributional ranges, they might be restricted to very different habitats and might have different phylogeographical histories. In the European Alps, our excellent knowledge of the evolutionary history of silicate-dwelling (silicicole) plants is contrasted by a virtual lack of data from limestone-dwelling (calcicole) plants. These two categories exhibit fundamentally different distribution patterns within the Alps and are expected to differ strongly with respect to their glacial history. The calcicole \textit{Ranunculus alpestris} group comprises three diploid species of alpine habitats. \textit{Ranunculus alpestris} s. str. is distributed over the southern European mountain system, while \textit{R. bilobus} and \textit{R. traunfellneri} are southern Alpine narrow endemics. To explore their phylogenetic relationships and phylogeographical history, we investigated the correlation between information given by nuclear and chloroplast DNA data. Analyses of amplified fragment length polymorphism fingerprints and \textit{matK} sequences gave incongruent results, indicative for reticulate evolution. Our data highlight historical episodes of range fragmentation and expansion, occasional long-distance dispersal and on-going gene flow as important processes shaping the genetic structure of the group. Genetic divergence, expressed as a rarity index (‘frequency-down-weighted marker values’) seems a better indicator of historical processes than patterns of genetic diversity, which rather mirror contemporary processes as connectivity of populations and population sizes. Three phylogeographical subgroups have been found within the \textit{R. alpestris} group, neither following taxonomy nor geography. Genetic heterogeneity in the Southern Alps contrasts with Northern Alpine uniformity. The Carpathians have been stepwise-colonised from the Eastern Alpine lineage, resulting in a marked diversity loss in the Southern Carpathians. The main divergence within the group, separating the ancestor of the two endemic species from \textit{R. alpestris} s. str., predates the Quaternary. Therefore, range shifts produced by palaeoclimatic oscillations seem to have acted on the genetic structure of \textit{R. alpestris} group on a more regional level, e.g. triggering an allopatric separation of \textit{R. traunfellneri} from \textit{R. bilobus}.

Keywords: AFLP, endemism, hybridisation, phylogeography, \textit{Ranunculus alpestris} group, refugia.

Received 14 April 2008; revision received 15 July 2008; accepted 17 July 2008

Introduction

Climatic fluctuations during the past 3 million years have not only had a significant impact on the distribution of
biota by enforcing massive range shifts and changes in abundance (Hewitt 2000, 2004), but also enforced speciation (Bennett 2004). For instance, for biota lacking adaptations for surviving in the tundra steppes prevailing in the lowlands of Central Europe during the Last Glacial Maximum (LGM; Frenzel et al. 1992), cold periods are thought to have fostered allopatric diversification recurrently as well as speciation through fragmentation of distribution ranges combined with local adaptation. Subsequent warmer periods led to hybridisation via secondary contact (Comes & Kadereit 1998; Kadereit et al. 2004). Signatures of these historical processes have thereby been imprinted and are often still reflected in the genetic structure of present-day biota (Hewitt 1996, 2000, 2004; Comes & Kadereit 1998, 2003). Recent molecular clues in combination with palaeo-environmental reconstruction have aided locating glacial refugia and tracing the routes of postglacial spread. Comparative studies across ecologically similar, but taxonomically unrelated taxa have partly revealed congruent patterns of glacial history, but unexpected incongruences have also been found (e.g. Soltis et al. 1997, 2006; Taberlet et al. 1998; Schönswetter et al. 2005). Such differences might have arisen due to chance events, but also due to intrinsic characteristics of species, such as breeding system, dispersal ability or adaptation to ecologically divergent habitats (e.g. Taberlet et al. 1998; Schönswetter et al. 2005).

The impact of the Pleistocene climatic oscillations on phylogeography and evolution of mountain plants has been extensively studied in the European Alps (for a review see Schönswetter et al. 2005), an area which functions as an ideal study system for research in the causes and consequences of patterns of genetic variation, phylogeography, and ecology. Most species of the Alps are, although often codistributed, either strictly calcicole or silicicole (i.e. restricted to calcareous or siliceous bedrock; Ozenda 1988), and the availability of a high number of studies of silicicole plants contrasts with the near absence of studies of calcicole plants with a sampling design comparable to that of silicicole species. In the taxa studied so far (Anthyllis montana, Kropf et al. 2002; Rumex nivalis Stehlik 2002; Erinus alpinus, Stehlik et al. 2002; Pritzelago alpina, Kropf et al. 2003; Dryas octopetala, Skrede et al. 2006), the sampling either covered only a part of the Alps or did not include enough populations to allow detailed phylogeographical inferences. Additionally, only Kropf et al. (2003) and Skrede et al. (2006) included samples from the Carpathians, from where detailed information about glacial refugia is still scarce (but see Mráz et al. 2007). Due to the uneven distribution of calcicole and siliceous bedrock especially in the Eastern Alps – a siliceous core being flanked by peripheral limestone ranges (Egger et al. 2000) – glacial histories are expected to be fundamentally different for both groups of species (Schönswetter et al. 2005). The *Ranunculus alpestris* group (Ranunculaceae) is almost exclusively restricted to calcareous bedrock, making it a good model to test hypotheses on the location of Pleistocene refugia for calcicole plants of the European Alps. In its most recent circumscription based on DNA sequence data (Paun et al. 2005) which corroborated previous morphological studies (e.g. Baltisberger & Müller 1981; Müller & Baltisberger 1984; Huber 1988; Baltisberger 1994), the group comprises three white-flowered species characterised by petaloid honey-leaves without nectary scale. They are distributed in sub-alpine to alpine limestone areas of the southern European mountain system (Fig. 1). *Ranunculus alpestris* L. is widespread from the Cordillera Cantabrica over Pyrenees, Alps, and Central Apennines to the Carpathians, whereas *R. bilobus* Bertol. and *R. traunfellneri* Hoppe are narrowly distributed endemics of the southeastern Alps. The *R. alpestris* group forms a strongly supported, early diverged lineage within *Ranunculus* that evolved already in the Early Miocene (Paun et al. 2005). Irrespective of the rather ancient origin of the group, diversification among *R. alpestris* s. str., *R. bilobus* and *R. traunfellneri* might have started as recently as 1.1 million years ago (Paun et al. 2005).

Here, we explore the phylogenetic and phylogeographical history of the *R. alpestris* group in the European Alps and the Carpathians, using amplified fragment length polymorphism (AFLP) and sequence variation of chloroplast DNA (cpDNA) towards evaluating the impact of Pleistocene climatic fluctuations on evolutionary divergence of a calcicole plant. In particular, we aim to answer the following questions:

1. Are the main genetic lineages within the *R. alpestris* group corresponding to taxonomical entities?
2. Are the phylogeographical structure and the pattern of genetic diversity in the Alps and the Carpathians explained by hypothesised Pleistocene refugia for calcicole plants (Schönswetter et al. 2005) and thus markedly different from those of silicicole species?
3. Is there evidence for lineage sorting or hybridisation, and how significant are consequences of gene flow in the evolution of this group?

**Materials and methods**

**The study species**

The three species constituting the *Ranunculus alpestris* group are diploid (2n = 16, e.g. Goepfert 1974) and their karyotype exhibits no significant difference (Müller & Baltisberger 1984). Crossing experiments between all pair combinations of the three species have produced fertile hybrids (Müller & Baltisberger 1984). The three species are mainly distinguished by the shape of the basal leaves (i.e. depth of incisions and shape of the leaf basis). They are perennial herbs restricted to similar habitats on calcareous
bedrock or (rarely) basic silicates above the treeline. *R. alpestris* is insect-pollinated (Schroeter 1926; Wertlen 2006) and self-incompatible (Baltisberger & Müller 1981; Müller & Baltisberger 1984). Gravity, snowmelt water and epizoochory are probably the main vectors of seed dispersal (Müller-Schneider 1986; Gerber et al. 2004). Adult plants produce tightly clustered ramets; however, no extensive vegetative proliferation occurs (Gerber et al. 2004).

**Sampling**

The three species were sampled across the Alps and Carpathians. Following the strategy adopted for the IntraBioDiv project (Gugerli et al. in press), leaf samples were collected from at least one locality in every second 12′ latitude × 20′ longitude regular grid cell based on Niklfeld (1971; Fig. 1, Appendices S1–S2). Three individuals per sampling locality (named hereafter population) were collected randomly at a distance of at least 10 m, and dried in silica gel. This sampling scheme resulted in 89 sampled populations (*R. alpestris*, 82 locations; *R. traunfellneri*, four; *R. bilobus*, three; Fig. 1, Appendix S1). Twenty-three plants (from 20 populations) were sampled and extracted twice to test the reproducibility of AFLP fingerprinting (Bonin et al. 2004). Two samples were used as replicates between PCR plates, and were replicated more than twice (in total, 51 replicates have been performed). Voucher specimens are deposited at the herbarium of the University of Vienna (WU).

**DNA extraction and AFLP fingerprinting**

Total genomic DNA was extracted from similar amounts of dried tissue (c. 10 µg) with the DNeasy 96 plant mini kit (QIAGEN) following the manufacturer’s protocol. AFLP profiles were generated following established procedures (Vos et al. 1995) with minor modifications (Gugerli et al. in press). Genomic DNA (c. 200 ng) was digested with 1 U MseI (New England BioLabs) and 5 U EcoRI (Promega) and ligated (with 1.2 U of T4 DNA-Ligase; Promega) to double-stranded adapters in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems) for 2.5 h at 37 °C. Preselective amplification was performed using primer pairs with a single or two selective nucleotides. Three selective primer combinations were chosen after a primer trial (fluorescent dye in brackets): EcoRI AAG (VIC)-MseI CATA; EcoRI ACA (6-FAM)-MseI CTGA; EcoRI ACC (NED)-MseI CAT. For each individual, 1.2 µL 6-FAM-, 2 µL VIC- and 3 µL NED-labelled, Sephadex purified, selective

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PCR products were combined with 0.2 μL GeneScan ROX 500 (PE Applied Biosystems) internal size standard and 9.8 μL formamide and run on a capillary sequencer ABI 3170 (Applied Biosystems). Blind samples were included to test for contamination (Bonin et al. 2004). Fragments in the range 65–500 bp were aligned with ABI PRISM GeneScan 2.1 Analysis Software (PE Applied Biosystems) and visualised, scored and exported as binary presence/absence matrix using Genographer 1.6 (available from http://hordeum.msu.montana.edu/genographer/).

**cpDNA sequencing**

Chloroplast DNA haplotype variation was explored to complement the information given by the mainly nuclear (Bussell et al. 2005) AFLPs. A part of the matK region, plus a part of the 3′ flanking trnK intron that were previously found to be variable in *Ranunculus* (Paun et al. 2005) were sequenced for 37 accessions representing the three species and the groups indicated by the AFLP analysis. After an initial screening that revealed the virtual lack of variation over large parts of the range of *R. alpestris* s.l., we examined in detail the pattern of cpDNA variation within the two endemic species *R. bilobus* and *R. traunfellneri*, and in southern and southwestern Alpine populations of *R. alpestris*. The plastid region was amplified and sequenced using the primers trnK345f and trnK3r according to Paun et al. (2005). GenBank Accession numbers of the obtained sequences are indicated in Appendix S1.

**Analysis of AFLP data**

All monomorphic fragments and the ones present/absent in all but one individual were removed from the data set to avoid biased parameter estimates (Bonin et al. 2004). Average gene diversity over loci (πw, in the following termed ‘genetic diversity’) corrected for small sample size and its standard deviation for both sampling and stochastic processes was computed for populations and phylogroups with Arlequin 3.0 (Excoffier et al. 2005), available at http://cmpg.unibe.ch/software/arlequin3. The same program was used to conduct analyses of molecular variance (AMOVA). We estimated the number of private fragments, defined as markers confined to a single population or a single phylogroup. We calculated a rarity index, i.e. a measure of population genetic divergence, by computing ‘frequency-down-weighted marker values’ per population or phylogroup (DW, Schönswetter & Tribsch 2005; in the following termed ‘rarity’) equivalent to range-down-weighted species values in historical biogeographical research (Crisp et al. 2001). For each population or phylogroup, the frequency of each AFLP marker in that population was divided by the number of occurrences of that particular marker in the total data set. Finally, these values were summed up and corrected for the total number of markers and individuals. DW is expected to be higher in those populations or groups that harbour a high number of rare markers and to be independent from sample size. R scripts to calculate DW and additional documentation are available from http://www.intrabiodiv.eu/spip.php?article77. In order to test whether molecular indices are related to historical patterns of refugia, based on the map of presumed glacial refugia published in Schönswetter et al. (2005) and given in Fig. 2 (a, d), each individual was denoted as sampled (i) in or very close to refugia including peripheral nunatak refugia below the LGM snow line (i.e. a thermal boundary connecting points above which snow does not melt in a climatically average year, Körner 2003), or (ii) in once-heavily glaciated areas within the LGM snow line. In order to test whether genetic diversity and rarity (πw and DW) were correlated with regional abundance of populations, frequency data of occurrences of the *Ranunculus* species per grid cell were used. These were gathered across the entire grid, including additional information on the number of occurrences (frequency) within 16 subsquares per cell [IntraBioDiv Consortium-Floristic Group, unpublished: The IntraBioDiv Floristic Database, version 4.0 (June 2007). Mapping the Distribution of High-Mountain Taxa of the Alps and Carpathians. Maintained at University of Vienna, Department of Biogeography]. Correlation coefficients between πw, DW, and frequency were calculated with SPSS 15.0.

A midpoint-rooted neighbour-joining (NJ) dendrogram based on the genetic distance of Nei & Li (1979) was generated and bootstrapped (Felsenstein 1985) using 1000 replicates with Treecon 1.3b (Van de Peer & De Wachter 1997). To visualise the pattern of population differentiation based on pairwise FST values computed with Arlequin 3.0, we constructed a principal coordinate analysis (PCoA) using the modules ‘Dcenter’ and ‘Eigen’ from NTSYS-pc (Rohlf 1997). Following Paun et al. (2006), we estimated the goodness of fit of the analysis by generating a model distance matrix from the eigenvector matrix (with ‘Simint’) and comparing it to the original FST matrix (using ‘Mxcomp’ and 1000 permutations).

Both spatial and nonspatial genetic mixture analyses implemented in the program Bayesian Analysis of Population Structure (baps 4.13, Corander et al. 2003, 2004, 2008; Corander & Marttinen 2006; available at http://web.abo.fi/fak/mnf//mate/jc/software/baps.html) were used to cluster individuals into ‘panmictic’ groups. baps was run with the maximal number of groups (K) set to 1–10 and each run was replicated three times. The partition with the highest log marginal likelihood was plotted onto the distribution map (Fig. 1). Another model-based clustering approach, implemented in Structure 2.0 (Pritchard et al. 2000), was used to test the results obtained from baps. The algorithm implemented in Structure, although developed for codominant markers, can be also used for
dominant markers under a ‘no admixture’ model. The number of clusters was estimated using 10^6 iterations, with a burn-in period of 10^5 iterations. The number of clusters ($K$) was used as a prior value; two replicates for each $K$ were analysed from $K = 1$ to $K = 10$. All analyses using Structure were carried out at the Bioportal of the University of Oslo (www.bioportal.uio.no).

Analysis of cpDNA sequence data

cpDNA sequences were edited using AutoAssembler 1.4.0 (Applied Biosystems) and manually aligned, together with accessions of *R. bilobus* and *R. traunfellneri* available in GenBank (part of Accessions AY954220 and AY954222). One informative 6-bp insertion/deletion (indel) from the
Table 1 Overview of genetic diversity and rarity estimates of the regional groups/gene pools/taxonomic entities investigated. N, number of population samples; \( \pi_n \), average gene diversity over loci; \( N_{\text{priv}} \), number of private AFLP markers; DW, within-population rarity of markers. Information on the groups is given in Fig. 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>( \pi_n )</th>
<th>( N_{\text{priv}} )</th>
<th>DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western Carpathians only</td>
<td>5</td>
<td>0.076 ± 0.039</td>
<td>4</td>
<td>0.789</td>
</tr>
<tr>
<td>Southern Carpathians only</td>
<td>2</td>
<td>0.012 ± 0.008</td>
<td>0</td>
<td>0.563</td>
</tr>
<tr>
<td>All Carpathian populations</td>
<td>7</td>
<td>0.073 ± 0.037</td>
<td>4</td>
<td>0.721</td>
</tr>
<tr>
<td>Eastern Alps only</td>
<td>55</td>
<td>0.102 ± 0.049</td>
<td>65</td>
<td>0.771</td>
</tr>
<tr>
<td>E phylogroup</td>
<td>62</td>
<td>0.102 ± 0.049</td>
<td>93</td>
<td>0.766</td>
</tr>
<tr>
<td>W phylogroup</td>
<td>16</td>
<td>0.080 ± 0.040</td>
<td>12</td>
<td>0.747</td>
</tr>
<tr>
<td>S phylogroup</td>
<td>11</td>
<td>0.134 ± 0.066</td>
<td>76</td>
<td>2.442</td>
</tr>
<tr>
<td>R. alpestris s. str.</td>
<td>82</td>
<td>0.108 ± 0.052</td>
<td>41</td>
<td>0.842</td>
</tr>
</tbody>
</table>

The trnK intron region was coded as present/absent, and the region itself was excluded from further analysis (following the recommendations of Simmons & Ochoterena 2000). A minimum-spanning tree (MST) of the haplotypes was drawn manually. Additionally, phylogenetic analyses were conducted using paup* 4.0b10 for Macintosh (Swofford 2003). Maximum-parsimony analyses were performed under a heuristic search strategy, using stepwise addition, tree-bisection–reconnection branch swapping, the MULTREES option on, and simple addition of sequences. The same options were selected for a nonparametric bootstrap analysis (Felsenstein 1985) with 1000 replicates. Sequences from R. aconitifolius, R. crenatus and R. parrassifolius ssp. parrassifolius (part of GenBank Accession nos AY954228, AY954217 and AY954224) were used for outgroup rooting in accordance with Hörandl et al. (2005) and Paun et al. (2005). The number of substitutions between the two major ingroup lineages in the amplified coding region was used to estimate the age of the respective split, based on a matK substitution rate calculated for Ranunculus of \( 2.2 \times 10^{-9} \) per site per year, with a standard deviation of \( 0.68 \times 10^{-9} \) (Paun et al. 2005).

Results

AFLP data

The three AFLP primer combinations yielded 438 clear polymorphic fragments. In the AFLP profiles from replicated samples, 46 differences were observed out of 13578 phenotypic comparisons, resulting in an error rate of 0.34%. All 263 individuals had different AFLP phenotypes. Average gene diversity over loci (\( \pi_n \); Appendix S1) varied from 0.003 in population 82 (Southern Carpathians) to 0.104 in population 52 (Northern Alps). Rarity (DW; Appendix S1) varied from 0.47 in population 25 (Central Alps) to 3.62 in population 83 (Ranunculus bilobus). Values of \( \pi_n \), DW and private fragments calculated for the phylogroups defined below are given in Table 1. Genetic diversity followed a clear north to south distribution with highest diversity in the Northern Alps and lowest diversity in the Southern Carpathians (Fig. 2a–c). In the Alps, rarity (DW) followed the opposite trend, with higher rarity in the Southern (but also northeastern) Alps and lower in the Central Alps and the Southern Carpathians (Fig. 2d–f). \( \pi_n \) and DW were not correlated (Pearson’s \( r = –0.184; P = 0.084 \)). For the Alpine populations, DW was significantly higher in potential refugia than in once-heavily glaciated areas inside the LGM snow line (Kruskal–Wallis test, \( \chi^2 = 13.147; P < 0.001 \)), whereas the pattern of \( \pi_n \) did not correlate with the location of potential refugia (\( \chi^2 = 1.801; P = 0.180 \)). Frequency of populations in a grid cell was significantly correlated with \( \pi_n \) (Spearman’s \( r = 0.311; P = 0.003 \)), but not with DW (Spearman’s \( r = –0.110; P = 0.310 \)).

In the NJ analysis (Fig. 3), three major phylogroups were separated with moderate or no bootstrap support. One group, termed phylogroup E in the following, is represented by Eastern Alpine R. alpestris (including the Carpathian populations), and another by the Western Alpine R. alpestris (phylogroup W). The relationships within these two groups were unresolved. A third phylogroup [S; bootstrap support (BS), 98%] comprised the clearly separated R. bilobus (BS, 98%), and R. traunfellneri (BS, 100%), plus four populations of R. alpestris from the S and SW Alps. Consequently, R. alpestris was not resolved as monophyletic in this analysis. The three-dimensional PCoA (Fig. 4) showed a congruent pattern with the NJ tree. The goodness-of-fit analysis for the scatter-plot indicated a matrix correlation value of \( r = 0.91 \) at \( P = 0.002 \) (one-tailed probability). A PCoA derived under the same conditions, but on a reduced data set including a comparable number of populations from each of the three groups, indicated a similar pattern (not shown).

The partition with the highest log marginal likelihood (special –26227.1, nonspecial –25852.3) produced by baps consisted of three clusters that corresponded to the three groups in the NJ analysis (Fig. 1). An identical grouping was indicated also by Structure. The likelihood values for \( K \) reached a clear plateau at \( K = 3 \) (Appendix S3); only ‘empty’ clusters (i.e. with < 0.1% membership) were added when \( K \geq 4 \).

AMOVA tests (Table 2) attributed 44.4% of the overall genetic variation to the among-population component. In nested AMOVA tests, the variation between the three species accounted for 29% and the variation among the three groups indicated by the NJ, PCoA, baps and Structure analyses for 20.1% of the overall variation. Lower values were found for the differentiation between phylogroups.
E and W (18.9%), and between the Alpine and the Carpathian samples of phylogroup E (12.6%). Two-level AMOVA tests calculated for each of the three phylogroups (E, W and S) indicated higher differentiation within the S group.

cpDNA sequence data

Eighteen substitutions out of 1422 aligned positions, plus one coded indel were parsimony-informative. Nine haplotypes have been identified within the 39 individual sequences. The method by Dixon (2006; online interface available at http://www.botanik.univie.ac.at/plantchorology/haplo.htm), which calculates a posterior probability distribution of the total number of haplotypes, including those which may have not been sampled, indicates a ‘very likely’ ($P = 84\%$) complete haplotype sampling, with a 95% confidence interval of (9, 10) haplotypes. A single most parsimonious tree (50 steps) was found (Fig. 5) with consistency index (CI) of 0.96 and retention index (RI) of 0.99. The *R. alpestris* group was resolved as monophyletic (BS, 96%). Within the ingroup, two main lineages were found, but none of the three species was monophyletic. *R. alpestris* and *R. bilobus* were partitioned to both clades.
The two cpDNA lineages differed by six point mutations (substitutions) out of 1179 bp sequenced from matK. The resulting sequence divergence for the main lineages pair of 0.509% would imply an average divergence time of 2 million years ago, i.e. within the interval 3.3–1.7 million years ago.

**Discussion**

*Diversification of the Ranunculus alpestris group*

Neither the two well-supported clades displayed by cpDNA sequences (Fig. 5) nor the three clusters of individuals evidenced by AFLPs (Figs 1 and 3–4) parallel the recognised taxa of the *Ranunculus alpestris* group. Incongruence between the two markers is caused by the populations at the southern border of the Alpine distribution, an area characterised by (i) the occurrence of all three species, (ii) high cpDNA variation compared to the rest of the distribution area (seven haplotypes from both main clades vs. two haplotypes from one clade), and (iii) the occurrence of strongly differentiated AFLP lineages (Fig. 3).
One of the main cpDNA lineages was restricted to *R. alpestris* and one population of *R. bilobus* (population 84), whereas the second main cpDNA lineage comprised accessions not only of *R. bilobus* and *R. traunfellneri*, but also two populations of *R. alpestris* from the southeastern Alps (Fig. 5). In the AFLP data, in contrast, *R. bilobus* and *R. traunfellneri* formed monophyletic entities, and only the placement of population 19 from the Southwestern Alps prevented *R. alpestris* from being resolved as monophyletic (Fig. 3).

Assuming a proper inference of the mutation rate of *matK* for *Ranunculus* (Paun et al. 2005), cpDNA suggests a main divergence event within the *R. alpestris* group during the Pliocene or Early Pleistocene. This correlates with evidence for some European alpine plants that had already started diversifying in the Late Tertiary or Early Quaternary (e.g. *Globularia*, *Soldanella*, *Gentiana* sect. *Ciminalis*, Kadereit et al. 2004). The split between *R. traunfellneri* and *R. bilobus* as well as the formation of phylolgroups E and W (see also below) that show no cpDNA haplotype divergence can be safely dated to the Pleistocene, as these events must have happened after the formation of the two cpDNA clades. With a probably complete haplotype sampling (see Results), the presence of a synapomorphic 6-bp deletion in the *atpK* intron in all individuals analysed of *R. traunfellneri* and its absence in population 83 of *R. bilobus* (Fig. 5) suggests an allopatric origin of the former from the latter. Recurrent isolation in refugia might have caused this allopatry and was suggested to have strongly fostered allopatric diversification and speciation also in other plant groups that radiated in the Alps (Comes & Kadereit 1998).

There are several potential explanations for the inconsistency between the information from cytoplasmic and nuclear signals, such as differential lineage sorting of ancestral polymorphisms in chloroplast and nuclear genes (Comes & Abbott 2001) or evolutionary convergence (i.e. homoplasy, Davis et al. 1998). The conflict between the present data derived from chloroplast and nuclear genomes, however, is most likely the result of recent chloroplast capture via hybridisation and introgression, as previously reported in numerous taxa such as *Eucalyptus*, *Gossypium*, *Helianthus*, *Pinus*, and *Quercus* (Rieseberg & Soltis 1991; Tsitron et al. 2003). Species of the *R. alpestris* group are able to hybridise as crossing experiments in all combinations of the three taxa were successful (Müller & Baltisberger 1984). Introgressive hybridisation after allopatric differentiation might have led to transfer of cpDNA from *R. alpestris* to *R. bilobus* in the case of population 84. The same, but in opposite direction, seems to apply to populations 19 and 20 of *R. alpestris*. Frequent local hybridisation is corroborated by the Bayesian cluster analysis of AFLP data (Fig. 1), which combined individuals from southern Alpine *R. alpestris* with *R. bilobus* and *R. traunfellneri* into one (‘panmictic’) gene pool. Mixing of previously isolated populations has long been related to Quaternary climatic changes (e.g. Stebbins’ ‘secondary contact’ model, Stebbins 1984). Independent evidence for hybridisation comes from population 85, which is morphologically intermediate between *R. bilobus* and *R. alpestris* (F. Prosser, personal communication), but genetically pertains to *R. bilobus*, as revealed by AFLPs (Fig. 3). In conclusion, our genetic data along with the reciprocal interfertility (Müller & Baltisberger 1984) suggest that the three investigated taxa should be best treated as subspecies, *R. alpestris* L. ssp. *alpestris*, *R. alpestris* L. ssp. *traunfellneri* (Hoppe) Nyman, and *R. alpestris* L. ssp. *bilobus* (Bertol.) Paun comb. nov. (= *R. bilobus* Bertol. in Misc. Bot. 19: 5, 1858, basionym). A thorough taxonomic study in the Southern and Southwestern Alps is necessary, because more subspecies might be still found there.

**Phylogeography of the *R. alpestris* group in the Alps: southern heterogeneity in refugia vs. northern uniformity**

Genetic variation and rarity, the latter measured as frequency-down-weighted marker values which highlight populations with rare markers, show a contrasting pattern in the *R. alpestris* group in the Alps. High rarity is found in potential refugia (Fig. 2d) whereas high genetic diversity is present in populations from the northern part of the Alps most of which has been fully glaciated, but where *R. alpestris* is very abundant, populations are large and well connected (Fig. 2a). Patterns of genetic variation seem strongly influenced by present factors such as actual population size which results in a correlation of diversity and regional abundance. Rare markers (e.g. ‘endemic’ fragments) representative for genetic divergence, appear to be a much better indicator of historical processes and are correlated with refugia (Comps et al. 2001; Widmer & Lexer 2001).

The heterogeneous southern/southwestern Alpine phylogroup (S, populations 17–20, and 83–89) is disjunctly but narrowly following the southern border of the Alps. This phylogroup includes all three taxa of the *R. alpestris* group. It corresponds well to known refugia, and nowadays, areas of endemism for vascular plants in the Alps (Pawlowski 1970; Tribsch 2004). The southern margin of the Alps has been previously suggested to be one of the main refugia for alpine plants depending on calcareous bedrock (e.g. Tribsch & Schönswetter 2003). Besides exhibiting stronger internal genetic substructure (Table 2; Fig. 3), this group is characterised by a high number of private and rare markers (Fig. 2d, Appendix S1) and high differentiation as a whole (76 of the 260 present fragments in this group are private, Table 1), but rather low levels of within-population genetic diversity (Fig. 2a). Therefore, we favour the view that the genetic pattern in the Southern Alps is mainly a result of diversification due to vicariance in multiple refugia.
Although hybridisation of lineages occurred (see above), historical episodic restriction to few high alpine areas produced an isolation effect resulting in high levels of rarity (Fig. 2d). After survival in multiple refugia, this group hardly contributed to postglacial colonisation of the Alps.

The western phylogroup (populations 1–16) has average levels of diversity and local differentiation (Table 2, Fig. 2a, d). As a group, it has the lowest differentiation (12 private fragments of 190 present, Table 1). The lack of supported substructure (Fig. 3) and the relatively evenly distributed patterns of rarity (Fig. 2d) do not give exact clues for elucidating its glacial history. The group might well have survived the last glaciation in a northern-Alpine peripheral refugium in central Switzerland (refugium V from Schönswetter et al. 2005), where we find at present increased levels of genetic variation (Fig. 2a, Appendix S1). From there, genetic diversity decreases in parallel to the presumed migration route towards southwest. A similar pattern has previously been found in Saponaria pumila in the eastern Central Alps (Tribsch et al. 2002). The northern Swiss Alps had long been postulated to represent a glacial refugium (Briquet 1906) and were suggested to have provided suitable habitats during the last glaciation for other species (Rumex nivalis, Stehlik 2002; Erinus alpinus, Stehlik et al. 2002) growing also on calcareous bedrock.

The eastern phylogroup (E, populations 21–82) is the geographically most widely distributed of the three AFLP groups, and is generally characterised by the presence of rather high levels of within-population genetic variation (Table 2, Fig. 2a, Appendix S1), and, as a phylogroup, by the highest number of private fragments (93 out of 345 present, Table 1). Both rarity and genetic diversity show higher levels in the north and east of the Alps and become lower towards the southwest. Moreover, a high level of gene flow among populations, and thus, rather weak differentiation compared to group S, is indicated by the AMOVA results (Table 2). This supports the classical view of an important northeastern Alpine refugium for calcicolous alpine plants that has been suggested based on palaeo-environmental data and patterns of endemism (see e.g. Merxmüller 1954; Tribsch & Schönswetter 2003; Tribsch 2004). Additionally, two localities at the northern border of the Alps (populations 38 and 52) show increased levels of rarity (Fig. 2d) and are located in formerly less-glaciated areas. Based on species’ distribution patterns, it was hypothesised earlier that small areas between glacier tongues have acted as refugia (Merxmüller 1954). Recently, the existence of glacial refugia for calcicolous plants at the northern border of the Alps has been indicated in two studies (Stehlik 2002; Stehlik et al. 2002; see also Schönswetter et al. 2005), one of which postulates a refugium in the Bavarian Alps for Rumex nivalis, close to population 38 from this study. It seems, however, that populations in refugia remained less isolated than in the southern part of the Alps and that rapid postglacial colonisation of Central and Eastern Alps from a main northeastern refugium has lead to a fairly uniform phyleogeographical pattern without internal support of populations/subgroups (Fig. 3).

The Carpathians were step-wise colonised from the Eastern Alps

Phylogroup E comprises also two disjunct areas in the Carpathians with a single cpDNA haplotype (the most common in R. alpestris, Fig. 5). AFLP data suggest that in an early colonisation event, R. alpestris reached the Tatra mountains in the Western Carpathians. The populations from the Tatras are not genetically bottlenecked, exhibit a level of rare markers similar to many Alpine populations (Fig. 2, Appendix S1), and are somewhat divergent from the Alpine populations of phylogroup E as illustrated by NJ (Fig. 3), PCoA (Fig. 4) as well as AMOVA (Table 2). A similar biogeographical history of Carpathian and Alpine populations has been suggested for another largely calcicole plant species, Pritzelago alpina (Kropf et al. 2003). Range expansion in the opposite direction, i.e. from the Tatras to the Eastern Alps, was previously suggested for the arctic-alpine Comastoma tenellum (Schönswetter et al. 2004), the rare arctic-alpine R. pygmaeus (Schönswetter et al. 2006) and the widespread Arabis alpina (Ehrich et al. 2007). Colonisation of the Alps from the Tatras appears, however, unlikely for R. alpestris given the (although unsupported) topology of the NJ dendrogram and the high number of private fragments in the Alpine part of the phylogroup E (65 vs. four in the Tatras, Table 1). The Western Carpathians were only partly glaciated during the Pleistocene, and are loosely connected with the Eastern Alps via a 300 km long ridge with elevations of up to c. 1000 m. Thus, a rather continuous distribution of alpine habitats during glacial periods appears possible. Moreover, a close biogeographical relationship is also documented by a large number of eastern Alpine-Carpathian endemics (e.g. Pawlowski 1970).

The isolated populations in the Southern Carpathians are nested within the samples from the Tatras as a distinct, genetically extremely depauperate group (Appendix S1) with almost absent genetic differentiation (Fig. 2, Table 1). Such a pattern indicates a long-distance dispersal from the Tatras to the Southern Carpathians in fairly recent times, probably during or even after the last glaciation, which was accompanied by a strong founder effect.

Altogether, our data suggest that historical processes mainly shaped allelic richness in the R. alpestris group, while genetic variation is rather reflecting present-day processes such as gene flow. Thus, in genetic fingerprinting studies, rarity should be used to unravel refugia rather than genetic diversity. Nevertheless, to fully understand plant evolution and speciation, both aspects have to been
seen in a framework of a changing environment caused by climatic fluctuations. To discriminate between hypotheses of ongoing speciation in historical refugia and of gene flow as driver for diversity, other approaches complementing a phylogeographical or phylogenetic approach are needed. Such approaches might involve comparison of the ecology of sympatric and parapatric populations or sister species in order to test, whether these populations are already adapted to their habitat or whether just drift in allopatric refugia has caused the neutral phylogeographical pattern.

Acknowledgements

We thank Thorsten Englisch for help with producing Fig. 1 and for providing frequency data from the unpublished IntraBioDiv floristic database. Harald Nikfled and Filippo Prosser are thanked for comments on earlier drafts of the manuscript especially on taxonomy, distribution of the taxa and corrections of toponymes. We are grateful to Christian Lexer, Elvira Hörandl and three anonymous referees for commenting on a previous version of the manuscript. Financial support from the European Commission for Science and Research, under the 6th Framework Programme (project STREP, SUSTDEV-2002-3.III.1.4: IntraBioDiv) and from the Austrian Science Fund (Erwin-Schrödinger fellowship to O.P., J6406-B03) is acknowledged.

References


This study was a small part of a biodiversity research programme (IntraBioDiv) in which genetic, species’, and habitat diversity in Alps and Carpathians was studied simultaneously. OP is mainly interested in plant evolution, with a special focus on polyploidy and special breeding systems. PS and AT have a broad interest in studying evolution, taxonomy, and biogeography of arctic and alpine plant species. MW currently focuses on plant population dynamics including population genetics.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Appendix S1** Details of the 89 populations of *Ranunculus alpestris* s.l. investigated in the present study.

**Appendix S2** Sampling localities codes indicated with numbers on a geographical map.

**Appendix S3** Log-likelihood values of each number of groups (K) as estimated by Structure 2.0 plotted against the K-values. Standard deviation of $L(K)$ between runs at a given K varied between 0 ($K = 2$) and 33.9 ($K = 9$).

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