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Glacial history of the alpine herb *Rumex nivalis* (Polygonaceae): a comparison of common phylogeographic methods with nested clade analysis[1]

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The glacial history of the alpine herb *Rumex nivalis* was investigated using amplified fragment length polymorphisms (AFLPs) and restriction fragment length polymorphisms with polymerase chain reaction (PCR-RFLPs) of cpDNA. Both traditional statistical methods widely applied in phylogeographic research and nested clade analysis were used. The AFLPs indicated little geographic structure probably due to the wind-pollinated reproductive system of the dioecious *R. nivalis*. Because cpDNA haplotypes exhibited distinct distributional patterns, correlation between AFLPs and PCR-RFLPs was low. The results of common statistical methods and of nested clade analysis were largely congruent. Both supported in situ survival of one group of common haplotypes in the Central Alps. For another group of common haplotypes, classical phylogeographic analyses gave strong evidence for survival in peripheral refugia at the northern alpine border, whereas this conclusion was not as clearly supported in the nested clade analysis. Nested clade analysis provided several detailed insights on past and ongoing populational demographic processes. Thus, it is a valuable tool in the phylogeographical analysis of haplotype data, but it should preferably be combined with other statistical analyses. In situations with low genetic variation in cpDNA, classical phylogeographic analytical tools on nuclear DNA will still be the methods of choice.

Key words: AFLP; glacial survival; methodological comparison; migration; nested clade analysis; nunataks; PCR-RFLPs of cpDNA; Pleistocene glaciation.

Nested clade analysis was developed to reconstruct the evolutionary history of populations by combining a cladogram of the genetic relationships among haplotypes within these populations and their geographic distribution (Templeton, Boerwinkle, and Sing, 1987). Population structure thereby can be separated from population history, if it is rigorously and objectively assessed (Templeton, Routman, and Phillips, 1995; Templeton, 1998). Thereby, nested clade analysis can discriminate between phylogeographic associations due to recurrent but restricted gene flow vs. historical events operating at the population level, such as past fragmentatian, colonization, or range expansion (Templeton, 1998). Haplotypes are first linked in a cladogram that portrays the evolutionary steps connecting them to one another. The accuracy of the following analysis depends on the absence of recombination in case of nuclear genomes and the reliability of the estimated cladogram topology (Templeton and Sing, 1993). A program for nested clade analysis of the geographic distribution of haplotypes has recently become available (GEODIS 2.0; Posada, Crandall, and Templeton, 2000; program available at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm). This has stimulated phylogeographic investigations of mitochondrial DNA in animals (mtDNA; e.g., Creer et al., 2001; Mardulyn, 2001; Seddon et al., 2001; Turgeon and Bernatchez, 2001) and of ribosomal DNA in fungi (James et al., 2001), whereas studies of cpDNA in plants using nested clade analysis are still scarce (e.g., Maskas and Cruzan, 2000). Currently, much research is aimed to elucidate specific phylogeographic processes that have shaped the genetic pattern of alpine plants of the European Alps and of the Arctic (Nordal and Jonsell, 1998; Widmer and Baltisberger, 1999; Gugerli and Holderegger, 2001; Hagen, Giese, and Brochmann, 2001; Stehlik, Schneller, and Bachmann, 2001, 2002; Stehlik, Tribsch, and Schönswetter, 2001; Zhang, Comes, and Kadereit, 2001; Holderegger, Stehlik, and Abbott, 2002). A broad array of ''traditional'' statistical approaches to analyze molecular genetic data has been used in these studies. Among the more frequently applied are cluster analyses or ordination methods (neighbor joining, UPGMA [unweighted pair group method using arithmetic averages], correspondence analysis), Mantel tests of the relationships of different classifications of individuals to geographic subunits (e.g., in populations, in regions), AMOVA (analysis of molecular variance), census of rare alleles in specific geographic subunits within a taxon, or methods to detect isolation by distance. Nested clade analysis seems to be very promising in that it unifies many characteristics of these traditional methods and, at the same time, appears to be more powerful in disentangling older from more recent population level processes (Posada, Crandall, and Templeton, 2000). Many phylogeographic investigations will probably use nested clade analysis in the near future. It is therefore of interest to apply nested clade analysis and more traditional methods to the same data and to see whether they will come to equivalent or complementary phylogeographic conclusions. Therefore, I subjected molecular genetic data on populations of the European endemic *Rumex nivalis* Hegetschw. (Polygonaceae) to both an array of classical statistics as used, e.g., in Stehlik, Schneller, and Bachmann (2001, 2002), and to nested clade analysis.

In phylogeographic investigations of the European alpine plants *Eritrichium nanum* (Boraginaceae) and *Erinus alpinus*

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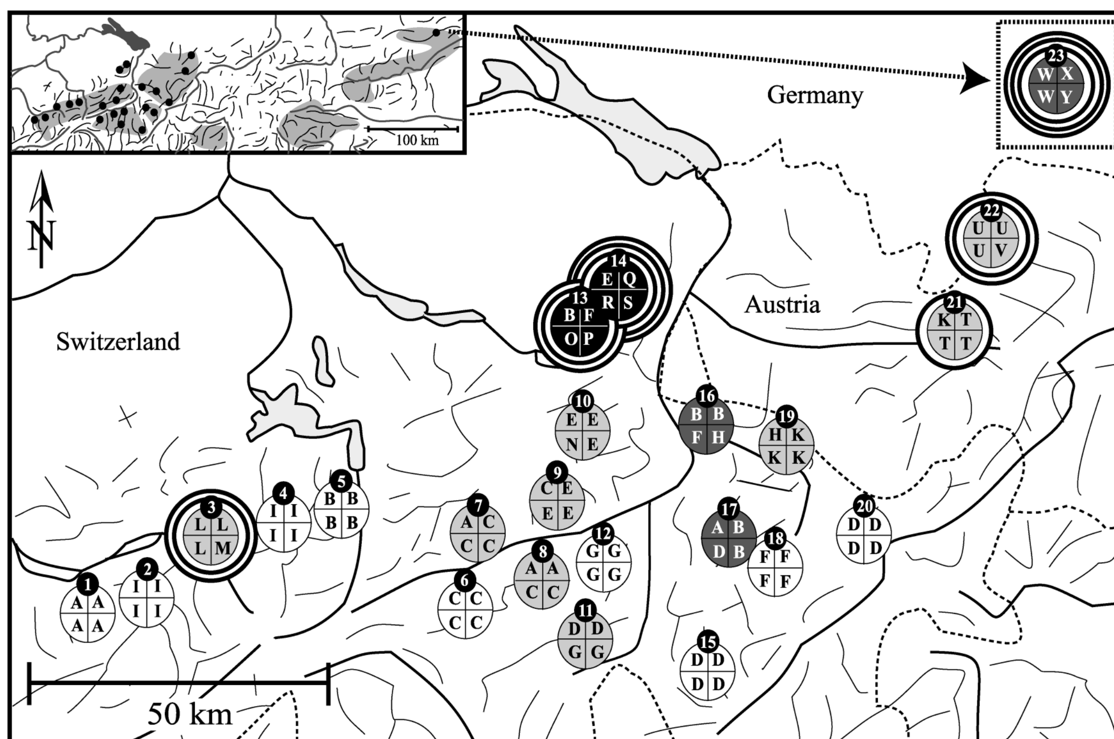


Fig. 1. The 23 populations sampled of *Rumex nivalis* in the Alps and the species' total distribution according to Meusel, Jäger, and Weinert (1965; inset). Twenty-two populations were sampled in the western part of the species' distribution, and one population from the eastern distribution limit was included for comparison. *Rumex nivalis* also occurs in Montenegro (Wagenitz, 1981). The letters indicate the haplotypes found in four plants from each population in the PCR-RFLP of cpDNA. Haplotypes were ordered alphabetically according to their frequencies and to their occurrence from west to east. Haplotypic richness (four to one haplotype) per population is indicated by a transgression from black to white. The number of private haplotypes per population is given by the number of circles around the populations. Dotted lines indicate national boarders.

(Scrophulariaceae), well-corroborated hypotheses for the species' response to the ice age glaciations could be tested on the basis of their present distribution patterns. By investigating mainly nuclear DNA (with amplified fragment length polymorphisms [AFLPs]; Stehlik, Schneller, and Bachmann, 2001, 2002) and cpDNA (with restriction fragment length polymorphisms using polymerase chain reaction [PCR-RFLPs] and sequence analysis; Stehlik et al., 2002), an in situ survival of *E. nanum* in several central alpine nunatak areas could be demonstrated. For the low-alpine *E. alpinus*, a postglacial immigration from a peripheral refugium in the Southern Alps was partly detected by AFLPs (Stehlik, Schneller, and Bachmann, 2002). However, a number of populations genetically strongly differentiated from this immigrant gene pool and is very likely to have survived in situ in a geographically restricted area at the northern border of the Alps. In the distribution pattern of *R. nivalis*, there is a thinning in population density towards the species' western distribution limit. Two possible explanations for this distribution pattern can be formulated. (1) Postglacial migration from (northeastern) peripheral refugia towards (the southwest of) its present distribution, with the disjunctions resulting from either long-distance dispersal or Holocene extinction (tabula rasa hypothesis; Stehlik, 2000). (2) Its high-alpine distribution and affinity to extreme habitats with short vegetation periods could also point to in situ glacial survival on nunataks (nunatak hypothesis). Hence, present distribution patterns suggest no clear hypothesis for the population history of *R. nivalis*. It can be predicted that these two processes would result in different genetic signatures. In the former there should be a reduction in genetic diversity with distance from the glacial refugia. In comparison, the nunatak hypothesis predicts very long periods of small, genetically isolated populations, which might have accumulated private mutations, as observed in *E. nanum* and *E. alpinus* (Stehlik, Schneller, and Bachmann, 2001, 2002; Stehlik et al., 2002).

The principal aim of the present study was to reveal the glacial population history of *R. nivalis*, and, at the same time, to compare the results of nested clade analysis and more traditional statistical approaches of the species' seemingly complex phylogeography.

MATERIALS AND METHODS

The species—The perennial herb *Rumex nivalis* is well defined by its small size, mostly unbranched inflorescence, a basal rosette, and stems with no or rarely up to two fleshy leaves (Wagenitz, 1981). The species is wind-pollinated and dioecious with $2n = 14$ for females and $2n = 15$ for males. *Rumex nivalis* grows on calcareous, wet soils in snowbeds or along creeks mainly above the timberline at 1600–2900 m above sea level (asl; Wagenitz, 1981). Its patchy distribution covers the middle and eastern parts of the Alps (Fig. 1).

Sampling—In the western part of the species' distribution, I collected leaf material of 22 populations and included one population from its eastern edge of occurrence for comparison (population 23; Table 1; Fig. 1). Twelve individuals were sampled per population, at distances of minimally 2 m or empty spaces among individuals to avoid multiple collections of ramets of the same genet, as *R. nivalis* builds up intermingling cushion-like patches (personal observation). The sampling included populations from potential northern al-

TABLE 1. Population numbering, location, elevation (above sea level), genetic diversity parameters, and haplotype composition of the 23 populations of *Rumex nivalis* sampled from the Alps. Per population, 12 individuals were investigated with AFLPs, four with PCR-RFLPs of cpDNA.

Population	Location	Elevation (m)	Coordinates (N/E)	N_f[a]	N_{pf}[b]	N_{rf}[c]	Haplotypes(s)
1	Mürren	2480	7°51′21.6″/46°34′03.2″	85.77	1	7	4 A
2	Grindelwald	2360	8°00′53.8″/46°40′21.3″	85.82	5	9	4 I
3	Meiringen	2330	8°15′01.1″/46°45′59.4″	89.08	0	9	3 L, M
4	Engelberg	2260	8°23′02.6″/46°46′44.3″	87.00	0	10	4 I
5	Surenen	2140	8°32′58.6″/46°50′15.69″	88.83	0	9	4 B
6	Greina	2200	9°00′43.9″/46°37′41.1″	87.08	2	9	4 C
7	Brigels	2400	9°02′28.6″/46°48′59.8″	91.42	4	16	1 A, 3 C
8	Vals	2360	9°07′56.4″/46°33′54.6″	84.08	1	9	2 A, 2 C
9	Flims	2100	9°13′32.6″/46°53′05.8″	87.92	0	13	C, 3 E
10	Spitzmeilen	2170	9°14′11.8″/47°01′59.7″	90.83	1	11	3 E, N
11	Nufenen	2500	9°13′43.4″/46°33′39.6″	88.08	0	10	2 D, 2 G
12	Safien	2360	9°17′21.3″/46°42′33.8″	88.33	2	16	4 G
13	Churfirsten	2220	9°19′04.4″/47°09′22.0″	89.43	0	9	B, F, O, P
14	Säntis	2100	9°21′54.8″/47°14′35.0″	89.00	0	14	E, Q, R, S
15	Bercla	2280	9°35′21.5″/46°29′31.9″	86.08	1	13	4 D
16	Hochwang	2300	9°37′45.3″/46°52′01.7″	88.83	1	15	2 B, F, H
17	Arosa	2400	9°38′01.2″/46°46′56.9″	87.75	0	4	A, 2 B, D
18	Davos	2400	9°50′18.7″/46°42′19.6″	89.36	0	7	4 F
19	Partnun	2100	9°51′53.5″/47°00′52.1″	91.08	1	11	H, 3 K
20	Albula	2300	9°50′48.4″/46°34′56.7″	91.00	0	9	4 D
21	St. Anton	2320	10°09′06″/47°13′00.0″	88.58	0	11	K, 3 T
22	Oberstdorf	1930	10°23′00″/47°23′50.0″	88.25	0	17	3 U, V
23	Hinterstoder	1805	14°42′10″/47°03′35.0″	89.75	6	9	2 W, X, Y

[a] N_f = mean total number of fragments (AFLPs).
[b] N_{pf} = number of private fragments confined to a single population (AFLPs).
[c] N_{rf} = number of rare fragments occurring in less than 10% of all individuals of the total sample set (AFLPs).

pine peripheral refugia (Stehlik, 2000; populations 13, 14, 22), as well as proposed central alpine nunatak areas (populations 17, 18; Table 1; Fig. 1). To detect potential (postglacial) migration routes, sampling of populations between the above areas was relatively dense.

Whenever possible, i.e., when plants were flowering or fruiting, I noted the gender of the collected individuals. Leaf material was dried in silica gel and stored at room temperature.

DNA isolation, AFLP, and PCR-RFLP of cpDNA—Genomic DNA was extracted and cleaned using the DNeasy extraction kit (QIAGEN, Basel, Switzerland) according to the manufacturer's instructions. As an additional step, all samples were cleaned and precipitated with sodium acetate and cold ethanol. In a few cases, when the DNA quality was insufficient for PCR, the Wizard purification system of Promega (Madison, Wisconsin, USA) was used to remove inhibiting secondary compounds.

The AFLP protocol was carried out following the procedure described by Vos et al. (1995) with minor modifications using fluorescent dye-labeled primers (Microsynth, Balgach, Switzerland) on an ABI 377 automated DNA sequencer (Applied Biosystems, Rotkreuz, Switzerland). A subsample of 26 individuals, spanning the total sample set, was screened with 24 primer pairs for clear and reproducible bands and variation within and among populations. Three primer pairs fulfilling these options were chosen for the analysis of the total sample set of 276 individuals (12 individuals per population). Genomic DNA (100 ng) was digested with the restriction enzymes *Eco*RI and *Mse*I and ligated to double-stranded *Eco*RI and *Mse*I adapters (Vos et al., 1995). The ligate was preamplified with the one base-selective primer EA (5′-GACTGCGTACCAATTCA-3′) and the nonselective primer M0 (5′-GATGAGTCCTGAGTAAG-3′) and subsequently selectively amplified using the three primer pairs EA+GG/M0+CAA, EA+GG/M0+CAC, and EA+CC/M0+CTC. The products were separated on 6% polyacrylamide gels with an internal size standard (GENESCAN ROX 500; Applied Biosystems) using GENESCAN 3.1 software (Applied Biosystems). Presence or absence of each fragment within each individual was scored and assembled as a binary data matrix in GENOGRAPHER 1.1.0 (Benham, 1999). Reproducibility was tested by repeating AFLP procedures in case of indistinct individual electropherograms and by running PCR products on different gels with identical size standards.

I used the same subset of 26 individuals as in the screening for the AFLPs to search for polymorphisms in the PCR-RFLP analysis of cpDNA with the universal primers of Demesure, Sodzi, and Petit (1995). The PCR amplifications of approximately 1.5 ng of genomic DNA per individual were carried out using the procedures described in Demesure, Sodzi, and Petit (1995). From the six primer pairs tested, the primer pairs *trn*H/*trn*K, *trn*K/*trn*K, *trn*S/*trn*fM, *psb*C/*trn*S, and *trn*D/*trn*T (Microsynth) yielded amplification products. Approximately 50 ng of the PCR product was restricted using nine restriction enzymes (four-cutters: *Alu*I, *Hae*III, *Hha*I, *Hpa*II, *Mse*I, *Rsa*I; six-cutters: *Bam*HI, *Eco*RI, *Hind*III; Amersham-Pharmacia-Biotech, Dübendorf, Switzerland; Takara Biomedical, Gennevilliers, France; Boehringer-Mannheim, Rotkreuz, Switzerland) under conditions recommended by the manufacturers. The fragments were separated and visualized on vertical 8% polyacrylamide gels stained with ethidium bromide (0.8 μL/mL). All of the primer pairs revealed at least one insertion/deletion (indel). Therefore, the total sample set in the PCR-RFLP analysis of cpDNA included four individuals per population (for a total of 92 individuals) by the five primer pairs given above. In order to ensure the identity of indels, five different restriction enzymes (*Alu*I, *Mse*I, *Bam*HI, *Hind*III, and *Eco*RI) were applied to the PCR products of each primer pair. The lengths of uncut PCR products and of restricted fragments was determined against 1-kb and 100-bp ladders (Amersham-Pharmacia-Biotech; BioRad, Reinach, Switzerland) using the analysis software embedded in GENEGENIUS (Syngene, Cambridge, UK). By that procedure, partial digests were detected and excluded.

Data analyses using traditional methods—Some statistical analyses were done for both marker systems (AMOVA, diversity measures, ''regional,'' and ''distance class'' Mantel tests), whereas others were applied to only one (correspondence analysis and ''populational'' Mantel tests for AFLPs; minimum spanning network and nested clade analysis for PCR-RFLPs of cpDNA; see below).

Several AMOVAs at different hierarchical levels were calculated from a matrix of squared Euclidean distances between all pairs of AFLP phenotypes

or PCR-RFLP haplotypes (Excoffier, Smouse, and Quattro, 1992; Stehlik, Schneller, and Bachmann, 2001). While the assignment of individuals to populations remained the same in all analyses, various groupings of populations into regions were used in order to determine which of these groupings would give the highest discrimination of genetic variance among regions. Additionally, the assignment of populations to groups in a respective marker system was also defined according to the grouping with the highest regional discrimination in the other marker system. These comparisons gave an additional assessment of the congruence between the two marker types. In all analyses, 9999 permutations were run to obtain test statistics using ARLEQUIN 2.0 (Excoffier, Schneider, and Roessli, 2000).

As AFLPs resolved all individuals as separate multilocus phenotypes, a correspondence analysis based on Euclidean distances (based on band presence and absence; Legendre and Legendre, 1998) between pairs of individuals was calculated to illustrate the overall similarities among individuals (NTSYS-PC 2.0; Rohlf, 1997). To characterize the relationships among cpDNA haplotypes, I applied the binary data matrix to the minimum spanning tree algorithm (NTSYS-PC 2.02i; Rohlf, 1997). The reliability of the resulting network was assessed by a cophenetic correlation coefficient as implemented with NTSYS-PC 2.02i (Rohlf, 1997).

Mantel tests were calculated to quantify the correlation between the Euclidean distance between pairs of individuals and their inter-individual distances for both AFLP and PCR-RFLP data sets (Legendre and Legendre, 1998; Stehlik, Schneller, and Bachmann, 2001). In all tests, the normalized Mantel r-statistic was computed with 999 permutations using R PACKAGE 4.0 (Casgrain and Legendre, 1999). Different specifically adapted geographic model matrices were used in the calculation of populational, regional, and distance class Mantel tests of AFLP-data, where all individuals had separate genotypes. Thirteen distance classes comprising similar numbers of pairwise comparisons were chosen: (1) 0 km (i.e., within populations); (2) $0 < d < 25$ km; (3) $25 \leq d < 40$ km; (4) $40 \leq d < 55$ km; (5) $55 \leq d < 65$ km; (6) $65 \leq d < 75$ km; (7) $75 \leq d < 85$ km; (8) $85 \leq d < 100$ km; (9) $100 \leq d < 130$ km; (10) $130 \leq d < 150$ km; (11) $150 \leq d < 180$ km; (12) $180 \leq d < 300$ km; (13) $300 \leq d < 763$ km. Details of the setup of respective model matrices are given in Stehlik, Schneller, and Bachmann (2001); probability of Mantel's r-values were sequentially Bonferroni-adjusted (Rice, 1989).

As a diversity measure, I extracted the total number of fragments (AFLPs) or haplotypes (PCR-RFLPs) per population, the number and distribution of "private" fragments or "private" haplotypes per population (i.e., those confined to a single population), and "rare fragments" (AFLPs only; Stehlik, Schneller, and Bachmann, 2001). Fragments were treated as rare when they occurred in less than 10% of individuals in the entire AFLP data set.

Nested clade analysis—I performed nested clade analysis using GEODIS 2.0 (Templeton, Boerwinkle, and Sing, 1987; Templeton and Sing, 1993; Posada, Crandall, and Templeton, 2000) on the minimum spanning network as calculated above. In a first step, I converted the haplotype network manually into a series of nested clades, using the rules defined in Templeton, Boerwinkle, and Sing (1987) and Templeton and Sing (1993). There were three possibilities to join the symmetrically stranded clade 2-3 at an internal position of the haplotype network in *R. nivalis* (see RESULTS) with another clade in the next higher hierarchy level (clades 3-1 to 3-3). However, all haplotypes from clade 2-3 were, in contrast to those of clades 3-1 and 3-3, at least three mutational steps apart from any haplotype in clade 3-2. Hence, to choose between clades 3-1 and 3-3, I used sample size as a tie breaker and joined the ambiguous clade to that clade with fewer observations (clade 3-3) as recommended by Alan R. Templeton (Washington University, personal communication). Alternatively, situations with symmetrically stranded clades could be resolved by giving more weight to indels than to site mutations (Templeton and Sing, 1993). However, in *R. nivalis*, only indels have been found in the cpDNA analysis (see RESULTS). By using geographic distances among populations, two main types of distances were calculated: (1) the "clade distance," D_c, which measures the geographic spread of haplotypes within a given clade; and (2) the "nested clade distance," D_n, which indicates how far haplotypes within a clade are apart from the haplotypes of the evolutionarily closest sister clades. An "interior-tip" statistic (I-T; calculated separately for D_c and D_n) was also estimated within each nested category as the average interior distance minus the average tip distance. This interior vs. tip contrast corresponds to a young vs. old contrast and, to a lesser extent, rare vs. common (Posada, Crandall, and Templeton, 2000). The significance of these statistics was estimated through a Monte Carlo procedure with 1000 random permutations (Templeton, Routman, and Phillips, 1995). The interpretation of the observed distance patterns was done using a revised version of the inference key published by Templeton, Routman, and Phillips (1995), available at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm.

RESULTS

AFLPs and PCR-RFLPs of cpDNA—In the AFLP analysis on *Rumex nivalis*, 205 clear and reproducible fragments were identified with three AFLP primer combinations, of which 24.9% were monomorphic. Twenty-one additional fragments had to be excluded because they were correlated with plant gender (I. Stehlik, unpublished data). The length of scored AFLP products ranged between 100 and 500 base pairs (bp), with a mean length of 223 bp. AFLPs distinguished all individuals as separate multilocus phenotypes.

No restriction site polymorphism was detected in the PCR-RFLP analysis of cpDNA. The primer combination *trn*K/*trn*K yielded six, *trn*H/*trn*K and *trn*S/*trn*fM five, *trn*D/*trn*T four, and *psb*C/*trn*S three different indels (23 in total), resulting in 24 different haplotypes, A to Y (Table 1).

Data analyses using traditional methods—The correlation between the PCR-RFLP and the AFLP data sets, based on pairwise Euclidean distances among individuals, was low (Mantel's $r = 0.12$, $P = 0.004$). By excluding the genetically isolated populations 22 and 23 from this analysis (see below), the correlation was even weaker, although still significantly positive ($r = 0.08$, $P = 0.008$). The two marker systems were therefore analyzed separately.

In the PCR-RFLPs of cpDNA, the most common haplotypes, A, B, C, and D, occurred in four populations, haplotypes E and F in three, and G, H, I, and K in two populations, whereas all other 14 haplotypes were confined to single populations (private haplotypes; Table 1; Fig. 1). Populations were either monomorphic or showed up to four different haplotypes in the four individuals investigated (Table 1; Fig. 1).

The cophenetic correlation coefficient indicated that the minimum spanning network was robust ($r = 0.8$, corresponding to a "good fit"; Rohlf, 1997). The most closely related pairs of haplotypes were separated by one to five indels, and the maximum number of mutational steps was 25 between haplotypes C and N (Fig. 2). Three of the four haplotypes that occurred in four populations (A, B, D) were positioned at internal nodes, while the fourth, haplotype C, was situated at the tip of a branch (Fig. 2). There was a significant decrease in both the total number of haplotypes and the number of private haplotypes per population with increasing geographic distance from the northern alpine border towards the Central Alps (Pearson correlation coefficients; number of haplotypes: $r = -0.47$, $P = 0.05$; number of private haplotypes: $r = -0.71$, $P = 0.01$; data did not significantly deviate from a normal distribution according to Kolmogorov-Smirnov tests; Fig. 1). This diversity decline from the northern peripheral parts of the Alps is, however, based on four individuals analyzed per population only. In contrast to this pattern in the cpDNA, there was no such correlation in the number of total, private, and

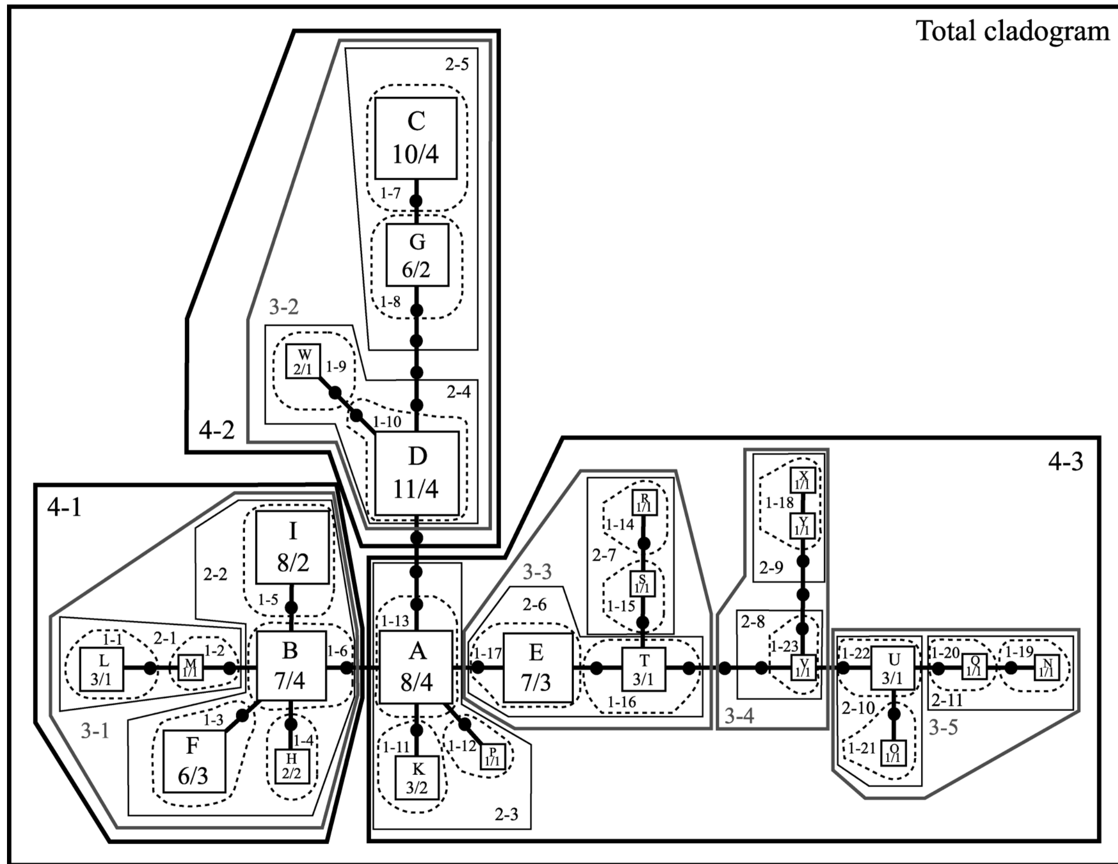
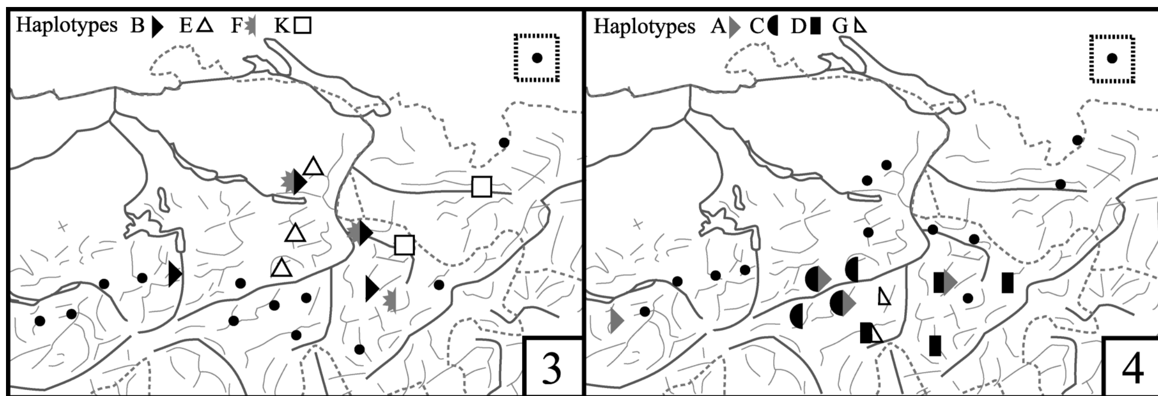


Fig. 2. CpDNA haplotype network based on indel differences among 24 haplotypes in 23 populations of *Rumex nivalis* in the Alps. The haplotype nesting design was subjected to a nested clade analysis (Table 3). The numbers below the identifying letter of each haplotype indicate the number of individuals representing a given haplotype and the number of populations in which this haplotype occurred. The size of the squares around haplotypes is proportional to their abundances. Black dots indicate linking haplotypes that were not detected in the PCR-RFLP analysis.

rare fragments in the AFLP data set ($P \geq 0.33$ in all cases). Moreover, there was no significant correlation among either of the above three diversity measures in the AFLP data ($P \geq 0.28$ in all cases).

There were two main types of geographic distribution patterns of haplotypes occurring in more than one population (Figs. 3, 4). (1) Haplotypes B, E, F, and K were detected in populations rich in private haplotypes (populations 13, 14, and 21) and, additionally, were distributed in populations near the northern border of the Alps (Figs. 1, 3). (2) Haplotypes A, C, D, and G were found in populations lacking private haplotypes and were geographically restricted to populations in or near the Central Alps (Figs. 1, 4). As an exception, haplotypes H and I, both occurring in two populations each, were found in



Figs. 3, 4. Distribution of two groups of haplotypes within 23 populations of *Rumex nivalis* in the Alps. **3.** Distribution of ''northern alpine'' haplotypes occurring in more than one population (haplotypes B, E, F, and K). **4.** Distribution of ''central alpine'' haplotypes occurring in more than one population (A, C, D, and G).

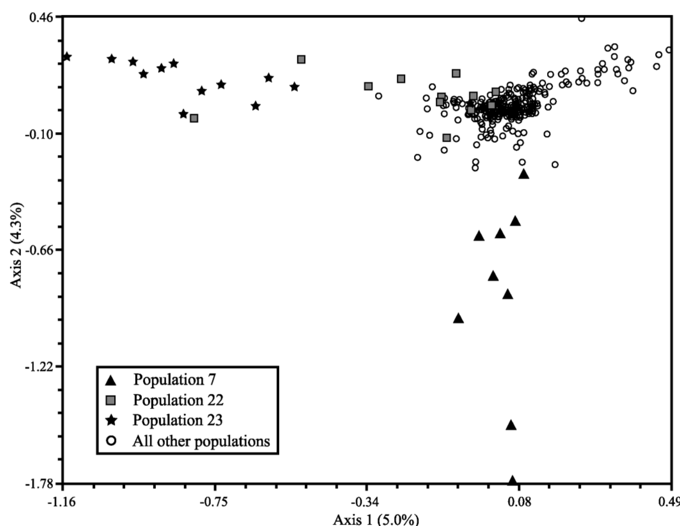


Fig. 5. Correspondence analysis of 23 populations of *Rumex nivalis* from the Alps based on AFLPs. Individuals of population 7 are labeled with filled triangles, individuals of population 22 with gray squares, individuals of population 23 with filled stars, whereas all other individuals are marked with open circles.

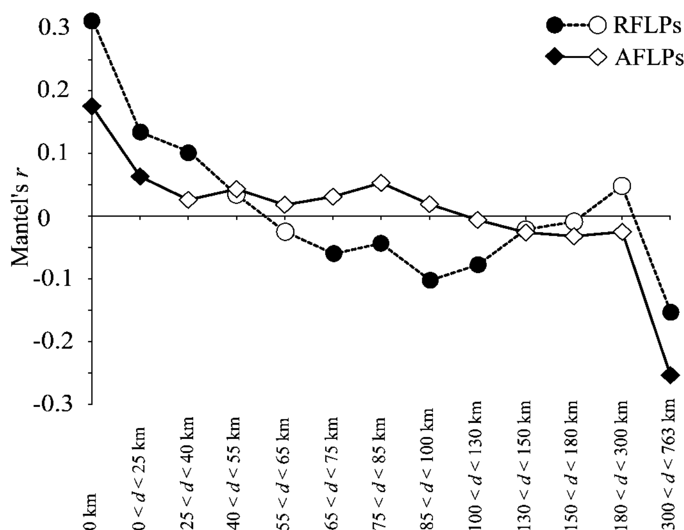


Fig. 6. Correlogram of Mantel's *r* values per distance class of 23 populations of *Rumex nivalis* from the Alps for both AFLPs (diamonds and solid lines) and PCR-RFLPs of cpDNA (circles and dashed lines). Filled symbols indicate Mantel's *r* values significantly different from zero at $P \leq 0.05$ after sequential Bonferroni adjustments.

populations lacking private haplotypes relatively near the northern Alpine border (Fig. 1).

The three first axes of the correspondence analysis of the AFLP data explained 5.0%, 4.3%, and 3.5% of the variation (totally 12.8%; Fig. 5). There was one main group with no internal geographic structure either with respect to populations or to regions, containing the majority of all sampled individuals of *R. nivalis* (populations 1 to 6 and 8 to 21; Fig. 5). All individuals of population 23 and many of population 22 were separated from the main group along the first axis, whereas many individuals of population 7 were separated along the second axis (Fig. 5).

Mantel tests of pairwise comparisons between populations based on Euclidean distances inferred from AFLPs (populational Mantel tests) showed almost no values that were significantly different from zero (data available from the author upon request). Significantly positive Mantel correlations occurred mainly among individuals within populations and between geographically neighboring populations, whereas significantly negative relationships were attributable to the genetic distinctness of individuals of populations 7, 22, and 23. None of these three latter populations showed significantly positive correlations with any other population. This situation is also reflected in the distance class Mantel test (Fig. 6). Significantly positive correlations for the AFLP markers were found up to a distance of 25 km (classes 1 and 2), and values were nonsignificant for the majority of classes at longer distances (i.e., class numbers 3 to 12). A significantly negative correlation was detected in the last distance class containing comparisons among individuals separated by distances between 300 and 736 km (Fig. 6). Nevertheless, when combining individuals from populations 1 to 21 to one region and populations 22 and 23 to a second region for a calculation of regional Mantel tests, the correlation among individuals within the first region was high (Mantel's $r = 0.29$, $P = 0.001$), while the correlation within the second region was nonsignificant. The Mantel correlation among individuals was significantly positive up to a distance of 40 km in the cpDNA data set and mainly remained significantly negative thereafter (Fig. 6). The regional correlogram was nevertheless similar to the one gained for AFLP (Fig. 6).

The AMOVAs clearly reflected the abovementioned different genetic structures in the AFLP and PCR-RFLP data sets (Table 2); a much higher percentage of variation was detected among populations in PCR-RFLP on cpDNA than in the AFLP data (63.94% vs. 20.97%; Table 2a, b). The grouping resulting in most discrimination among regions consisted of two groups in AFLPs (populations 1 to 21 and 22 to 23; Table 2c) and of six groups in PCR-RFLPs, paralleling the groupings of the correspondence analysis (Fig. 5) and the minimum spanning tree, respectively (Table 2e; Fig. 2).

Nested clade analysis—In the nested clade analysis, significant geographic associations of haplotypes were found mainly on the left side of the network (Table 3; Fig. 2; a detailed list of D_c, D_n, and I-T values including *P* levels can be obtained from the author upon request). Mainly, there was no geographic variation within clades 2-7 to 2-11 and 3-3 to 3-5 (on the right side of the haplotype network), as all haplotypes except E occurred in only one population (Figs. 1, 2). The null hypothesis of no geographic association of haplotypes was rejected only for clade 4-3. On the left side of the haplotype network, however, all clades formed significant geographic associations (except clade 2-1 due to lack of geographic variation; Table 3; Fig. 2). In the total cladogram, geographic associations of haplotypes were again nonsignificant. Three main types of historical processes acting on populations were detected (Table 3): (1) restricted gene flow with isolation by distance in most cases (clades 2-2, 3-1, 3-2, and 4-3); (2) (continuous) range expansion for clades 2-4 to 2-6; and (3) a past fragmentation for clade 2-3.

DISCUSSION

Congruence between AFLP and PCR-RFLP markers—The weak, but significantly positive correlation of the AFLP

TABLE 2. Results of analysis of molecular variance (AMOVA) of AFLP and cpDNA PCR-RFLP data from 23 populations of *Rumex nivalis* from the Alps. To compare the overall magnitude of variance among individuals between the two marker types, variance components are presented without grouping populations to regions (a, b). Groupings with different numbers of regions were determined to minimize the percentage of variation among individuals and to maximize the variation among regions and populations for each marker type. For those groupings that resulted in most divergence among regions per marker (c for AFLPs, e for PCR-RFLPs), corresponding values of the other marker type are also given (d, f).

	Grouping (population number)[a]	Source of variation	df	Percentage of variation
(a) AFLPs	(1–23)	Among populations	22	20.97***
		Within populations	252	79.03***
(b) PCR-RFLPs	(1–23)	Among populations	22	63.94***
		Within populations	69	36.06***
(c) AFLPs	(1–21), (22, 23)	Among regions	1	12.29***
		Among populations	21	16.80***
		Among individuals	252	70.91***
(d) PCR-RFLPs	(1–21), (22, 23)	Among regions	1	15.43***
		Among populations	21	53.15***
		Among individuals	69	31.42***
(e) PCR-RFLPs	(1, 3, 5, 12, 13, 16, 18, 19), (2, 4), (6–9), (10, 14, 21), (11, 15, 17, 20), (22, 23)	Among regions	5	32.74***
		Among populations	17	33.29***
		Among individuals	69	33.96***
(f) AFLPs	(1, 3, 5, 12, 13, 16, 18, 19), (2, 4), (6–9), (10, 14, 21), (11, 15, 17, 20), (22, 23)	Among regions	5	2.76***
		Among populations	17	18.61***
		Among individuals	252	78.63***

[a] For numbering and characteristics of populations see Table 1.
*** $P < 0.001$.

and PCR-RFLP data sets of the alpine *Rumex nivalis* showed two common basic characteristics. (1) The eastern Austrian population 23 and, to a lesser extent, population 22 in southern Germany were genetically distinct from all other populations. This was illustrated by the drop of overall Mantel correlations from 0.12 to 0.08 when populations 22 and 23 where excluded from the analysis. Next to the genetic distinctness of populations 22 and 23 in both marker types, population 7 was divergent in the AFLP marker set only. This genetic distinctness could not be explained by the proposed processes having shaped the genetic patterns of all other populations (see below). However, reasons for the special genetic position of population 7 among all populations could be an accumulation of mutations or a hybridization with a co-occurring *Rumex* species, although *R. nivalis* is stated never to hybridize with any other *Rumex* species (Wagenitz, 1981) and the plants in population 7 were not divergent based on morphological characteristics (personal observation). (2) Highest genetic correlations were found within populations and among geographically closest populations in both data sets (Fig. 6). However, in the AMOVAs, the best regional discrimination in AFLPs was detected when populations were partitioned into two groups (12.29%; same grouping for PCR-RFLPs: 15.43%; Table 2c, d), whereas the best discrimination in PCR-RFLPs was found with six population groups (32.74%; AFLPs: only 2.76%; Table 2e, f). While in the AFLP data set, Mantel correlations among individuals separated by distances between 25 and 300 km were never significantly different from zero (Fig. 6), Mantel analyses of PCR-RFLPs partly supported significantly negative genetic relationships between individuals separated by distances greater than 55 km (Fig. 6). The divergent genetic patterns between AFLPs and PCR-RFLPs are probably due to

TABLE 3. Nested contingency analysis of geographic associations and their interpretations according to Templeton, Routman, and Phillips (1995; GEODIS 2.0, available at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm) for 23 populations of *Rumex nivalis* in the Alps.

Clade[a]	Chi-square statistic	P	Chain of inference	Inferred demographic event
2–2	56.25	<0.000*	1-2-3-5-6-7-Yes	Restricted gene flow or dispersal but with some long-distance dispersal
2-3	26.00	0.002*	1-2-3-4-9-No	Past fragmentation
2-4	13.00	0.022*	1-2-11-12-No	Continuous range expansion
2-5	16.00	<0.000*	1-2-11-12-No	Continuous range expansion
2-6	10.00	0.029*	1-2-11-Yes	Range expansion
2-10	4.00	0.25	1-No	No geographic association of haplotypes
2-11	2.00	1.00	1-No	No geographic association of haplotypes
3-1	0.00	<0.000*	1-2-3-4-No	Restricted gene flow with isolation by distance
3-2	24.96	<0.000*	1-2-3-4-No	Restricted gene flow with isolation by distance
3-3	7.20	0.19	1-No	No geographic association of haplotypes
3-4	3.00	0.33	1-No	No geographic association of haplotypes
3-5	0.00	0.23	1-No	No geographic association of haplotypes
4-3	0.00	<0.000*	1-2-3-4-No	Restricted gene flow with isolation by distance
Total cladogram	145.56	<0.000*	1-No	No geographic association of haplotypes

[a] Clades not listed were not included in the calculation due to single haplotype occurrence and/or no geographic variation within clades. Nesting of clades followed Fig. 2.
* Significant at the 0.05 level using 1000 permutations.

the different transmission of nDNA (biparental) vs. cpDNA (uniparental and, as in most angiosperms, putatively maternal) and suggest restricted gene flow by seed (for pollen gene flow, see below). However, when comparing the level of geographic structure in the AFLP data found in the present study with that found in other alpine species investigated with AFLPs at similar spatial scales, the low geographic structure in *R. nivalis* becomes apparent. *Eritrichium nanum*, *Androsace alpina*, and *Erinus alpinus* (Stehlik, Schneller, and Bachmann, 2001, 2002; Stehlik, Tribsch, and Schönswetter, 2001) all show higher portions of genetic variation explained by correspondence analyses, higher percentages of genetic variation among populations or regions in AMOVAs, and/or more values significantly different from zero in Mantel analyses. Even *Saxifraga oppositifolia*, otherwise characterized by low genetic variation among populations and no particular geographic patterns in RAPD data both in the Alps and the Arctic (Gabrielsen et al., 1997; Gugerli, Eichenberger, and Schneller, 1999; Holderegger, Stehlik, and Abbott, 2002), showed slightly more geographic structure than did *R. nivalis* with AFLPs. The most obvious difference between *R. nivalis* and these four species is the breeding system. While *R. nivalis* is wind-pollinated, the others are pollinated by insects (Wagenitz, 1981; Gugerli, 1997; Stehlik, Tribsch, and Schönswetter, 2001; Stehlik, Schneller, and Bachmann, 2001; Stehlik et al., 2002). As summarized by Hamrick and Godt (1996), wind-pollinated and outbreeding species, such as the dioecious *R. nivalis*, show lower portions of total genetic variation among populations than do species with other breeding systems. All statistical analyses on *R. nivalis* revealed a picture of one large nuclear gene pool, connected by random gene flow by pollen, comprising populations 1 to 21 with the exception of population 7. No overall pollen : seed flow ratios (Ennos, 1994) could be calculated for *R. nivalis*, since gender proportions were strongly divergent from a 50 : 50 ratio and showed altitude dependent variation (I. Stehlik, unpublished manuscript). Nevertheless, wind pollination probably increased the difference between the main group of AFLP genotypes, which showed almost no geographic structure, and the strongly structured, putatively maternally inherited cpDNA haplotypes.

Comparison of traditionally used phylogenetic analyses with nested clade analysis—As most populations showed almost no geographic structure when analyzed with AFLPs, no conclusions on glacial refugia or migration routes could be inferred. Therefore, the historical interpretation of molecular data of *R. nivalis* relied on cpDNA haplotypes. This also enabled a direct comparison of the traditional statistical methods with nested clade analysis, as the latter method can be applied to haplotypic data only.

The genetic variation in the cpDNA was surprisingly high in *R. nivalis* (24 haplotypes). In *E. nanum*, Stehlik et al. (2002) distinguished 11 haplotypes in 37 populations covering the entire Alps. Holderegger, Stehlik, and Abbott (2002) found only four haplotypes in 15 populations in alpine *S. oppositifolia*, while Abbott et al. (2000) identified 14 haplotypes in the same species at the circumpolar level. Levels similar to *R. nivalis* were detected in *Dryas integrifolia* (20 haplotypes), but this sampling included the whole of North America (Tremblay and Schoen, 1999). In the present study, the majority of the investigated populations were sampled within a radius of approximately 150 km (Fig. 1), and the high haplotype variation was strongly geographically structured. AMOVAs yielding highest regional differentiation (Table 2e) comprised, among the totally six groups, two geographically restricted and coherent, mainly central alpine groups (populations 6–8, and 12; and 11, 15, 20, and 23), whereas the other four groups of populations were either overlapping or characterized by disjunctions (Fig. 1). It is then not surprising that significantly positive Mantel correlations were only detected among individuals at distances of less than 40 km (Fig. 6). Beyond this distance, populations were isolated by distance with only occasional gene flow by seed. Populations at and near the northern border of the Alps were significantly richer in both the total number of haplotypes and the number of private haplotypes (Fig. 1). This pattern was also reflected in the group of haplotypes B, E, F, and K occurring in the haplotypically rich populations 13, 14, 21, and 22. Otherwise these haplotypes only occurred in neighboring populations of these haplotypically rich populations (Figs. 1, 3). The occurrence of the private haplotypes L and M in population 3 near the northern border of the Alps also fit this pattern (Fig. 1).

This limited gene flow by seed, the patchy distribution of haplotypes, the high haplotype diversity, and number of private haplotypes at the northern periphery of the Alps support the glacial survival of *R. nivalis* in northern peripheral refugia. Populations 13, 14, and 22 are located in previously postulated peripheral refugia, whereas populations 3 and 21 are in close vicinity to these refugia (Stehlik, 2000). Although populations 13 and 14 were separated by only 11 km, they shared no haplotypes (at least based on the sample size of four individuals per population). The thinning of haplotype diversity towards the Central Alps and the wide distribution of haplotypes B, E, F, and K in the northern Alps and further south suggested that the peripheral refugial populations 13, 14, and 21 have acted as sources for the colonization of populations towards the Central Alps. However, not all refugial populations have contributed to this colonization, as northern Alpine populations 3 and 22 did not share any haplotype with another population.

The group of haplotypes C, G, and D (including haplotype W from the eastern-most sampled population) was most distant from all other haplotypes at the left side of the haplotype network (Fig. 2). These three haplotypes were also confined to a geographically restricted region in the Central Alps (populations 6–9, 11, 12, 15, 17, and 20; Figs. 1, 4). Haplotype A co-occurred with haplotypes C and D in certain populations, and only its occurrence in population 1 did not fit the central alpine distribution of the C-G-D group (Figs. 1, 4). Haplotype A was also closely related to the C-G-D group (Fig. 2). Judging from the regional distribution of haplotypes A, C, D, and G, nunatak survival of *R. nivalis* is plausible in the area comprising populations 6–8, 11, 12, 15, and/or 20 (Figs. 1, 4; for populations 9, 17, and 19 see below). This central alpine region was formerly not recognized as a nunatak area (Stehlik, 2000). Based on the distribution of the haplotypes, it could not be decided whether this pattern was due to glacial survival in several populations or to postglacial gene flow via seed within this central alpine area.

In addition to this proposed scenario of a glacial survival of populations of *R. nivalis* both in northern (populations 3, 13, 14, 21, and 22) and in central alpine locations (populations 6–8, 11, 12, and/or 15), a third pattern was suggested by the distribution of haplotypes H and I (Fig. 1). Both haplotypes were closely related to haplotype B (two mutational steps), whereas the mean number of mutational steps among directly

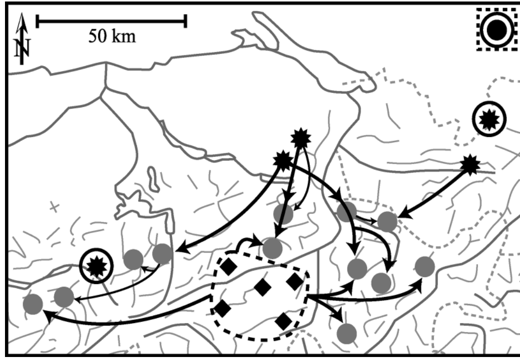


Fig. 7. Glacial and post-glacial history of *Rumex nivalis* in the Alps inferred from PCR-RFLPs of cpDNA. Stars represent populations that survived glaciation in peripheral refugia at the northern border of the Alps. Diamonds stand for in situ surviving populations in a central alpine nunatak area (surrounded by a broken line). Gray-circle populations were probably postglacially colonized. Thick arrows indicate the inferred migration routes out of peripheral refugia or central alpine nunataks, whereas thin arrows imply mutational changes along with these colonizations. No dispersal events originated in the three encircled populations.

related haplotypes was 3.5 (Fig. 2). The geographic restriction of haplotypes H and I could be interpreted as dispersal of haplotype B from the refugial population 13 into populations 5 and 16, with subsequent mutations leading to haplotype I in population 4 and to haplotype H in population 16. Haplotype I would then have further dispersed into population 2 and haplotype H into population 19 (Fig. 7). The available data and analytic tools do not allow further testing of this hypothesis.

Based on a summary of the interpretation presented so far (Fig. 7), it is clear that this traditional approach is to a certain extent a weak indicator of the glacial history of *R. nivalis*. Some populations rich in haplotypes, such as populations 9, 17, or 19, have to be regarded as sink areas between different sources of recolonization from populations 13, 14, 21, and from the central alpine nunatak region (Fig. 7). Can nested clade analysis help to solve such interpretational problems, and are its results concordant with those of a traditional statistical analysis?

At the lowest hierarchical level with within-clade geographic and genetic variation, haplotypes of clades 2-2 to 2-6 were characterized by significant geographic associations (Table 3; Fig. 2). In contrast, the null hypothesis of no geographic association of haplotypes could not be rejected for clades 2-10 and 2-11 (Table 3; Fig. 2). The haplotypes of these second-level clades were influenced by a broad array of demographic events. For instance, haplotypes of clade 2-2 were dispersed over short distances (restricted gene flow), although there was support for some long-distance gene flow as well (Table 3). With this background and knowing that population 13 (harboring haplotypes B and F) was located within a proposed northern alpine peripheral refugium, small-scale gene flow from the north to the southeast and a long-distance dispersal event into population 5 in southwestern direction could be inferred (Figs. 1, 3, 7). The distribution area of haplotypes A, K, and P (clade 2-3) was subjected to a past fragmentation (Table 3), which could easily be attributed to the influence of Pleistocene glaciers. Nested clade analysis provided more detailed information on possible past demographic events for this group of related haplotypes than the classical methods. Within the whole data set, only haplotypes C, G, D, and W of clades 2-4 and 2-5 were characterized by a continuous range expansion (Table 3). These haplotypes were also combined in higher level clades (3-2, 4-2; Table 3) and thereby separated from all other haplotypes (Fig. 2). When accepting that these haplotypes survived in situ within the Central Alps (or in eastern Austria in the case of haplotype W), this could illustrate a high colonizing capacity of haplotypes C, G, and D or a long-lasting range expansion after glaciation. On the other hand, all haplotypes characterized by restricted gene flow were situated at or near to the periphery of the Alps. This finding was again in concordance with the results of the classical analyses, but nested clade analysis provided a more detailed insight on past and ongoing events in *R. nivalis*. A more recent range expansion was more likely for haplotype E in clade 2-6 (Table 3; Figs. 1, 3, 7), possibly originating in the Central Alps, similarly to haplotypes C, D, and G. In the classical analyses presented above, haplotype E was inferred to have survived at the northern Alpine periphery and to have subsequently migrated into the Central Alps. At higher nesting levels, either geographic associations among haplotypes were nonsignificant (clades 3-3, 3-4, and 3-5) or restricted gene flow with isolation by distance was indicated (clades 3-1, 3-2, and 4-3; Table 3). The lack of a geographic association of the haplotypes in the total cladogram was probably caused by the broad variety of demographic events acting on the populations within clades 4-1 and 4-2 in a geographically restricted range and by the rarity and only limited relatedness of private haplotypes in clade 4-3.

Conclusions—The AFLP analysis of 23 populations in *R. nivalis* revealed a low geographic structuring of the 276 individuals investigated and a large nuclear gene pool connected by random gene flow via pollen. This agrees with the wind-pollinated and outbreeding reproductive system of this dioecious species. In contrast, PCR-RFLPs of cpDNA resulted in 24 different and geographically grouped haplotypes. The phylogeographic analyses of cpDNA in *R. nivalis*, including the comparison of statistical methods commonly used in phylogeographic investigations and of nested clade analysis, hitherto rarely applied in plants, were largely congruent. Both supported in situ survival of a group of common haplotypes in the Central Alps. For a second set of common haplotypes, classical phylogeographic analyses gave evidence for peripheral glacial refugia at the border of the Northern Alps (Fig. 7), whereas this conclusion was weaker supported by nested clade analysis. However, in many cases, nested clade analysis provided more detailed insights on past and ongoing populational demographic events, because it could discriminate among restricted gene flow and (continuous) range expansion (Table 3), processes that act at different spatial and temporal scales. Therefore, nested clade analysis is a valuable tool in phylogeographic research that displays its highest power when used together with other phylogeographic statistical methods. In situations with low genetic variation in cpDNA, classical phylogeographic analytical tools on nDNA will still be the methods of choice.

LITERATURE CITED

ABBOTT, R. J., L. C. SMITH, R. I. MILNE, R. M. M. CRAWFORD, K. WOLFF, AND J. BALFOUR. 2000. Molecular analysis of plant migration and refugia in the Arctic. *Science* 289: 1343–1346.

BENHAM, J. J. 1999. GENOGRAPHER. Vers. 1.1.0. http://hordeum.oscs.montana.edu/genographer.

CASGRAIN, P., AND P. LEGENDRE. 1999. The R PACKAGE for multivariate and spatial analysis. Version 4.0. User's manual. Department of Biological Sciences, University of Montreal, Montreal, Quebec, Canada.
CREER, S., A. MALHOTRA, R. S. THORPE, AND W.-H. CHOU. 2001. Multiple causation of phylogeographical pattern as revealed by nested clade analysis of the bamboo viper (*Trimeresurus stejnegeri*) within Taiwan. *Molecular Ecology* 10: 1967–1981.
DEMESURE, B., N. SODZI, AND R. J. PETIT. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology* 4: 129–131.
ENNOS, R. A. 1994. Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* 72: 250–259.
EXCOFFIER, L., S. SCHNEIDER, AND D. ROESSLI. 2000. ARLEQUIN Version 2.0. http://lgb.unige.ch/arlequin/.
EXCOFFIER, L., P. E. SMOUSE, AND J. M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.
GABRIELSEN, T. M., K. BACHMANN, K. S. JAKOBSON, AND C. BROCHMANN. 1997. Glacial survival does not matter: RAPD phylogeography of Nordic *Saxifraga oppositifolia*. *Molecular Ecology* 6: 831–842.
GUGERLI, F. 1997. Sexual reproduction in *Saxifraga oppositifolia* L. and *S. biflora* All. (Saxifragaceae) in populations of the Alps. *International Journal of Plant Sciences* 158: 274–281.
GUGERLI, F., K. EICHENBERGER, AND J. J. SCHNELLER. 1999. Promiscuity in populations of the cushion plant *Saxifraga oppositifolia* in the Swiss Alps as inferred from random amplified polymorphic DNA (RAPD). *Molecular Ecology* 8: 453–461.
GUGERLI, F., AND R. HOLDEREGGER. 2001. Nunatak survival, *tabula rasa* and the influence of the Pleistocene ice-ages on plant evolution in mountain areas. *Trends in Plant Science* 9: 397–398.
HAGEN, A. R., H. GIESE, AND C. BROCHMANN. 2001. Trans-Atlantic dispersal and phylogeography of *Cerastium arcticum* (Caryophyllaceae) inferred from RAPD and SCAR markers. *American Journal of Botany* 88: 103–112.
HAMRICK, J. L., AND M. J. W. GODT. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London Series B* 351: 1291–1298.
HOLDEREGGER, R., I. STEHLIK, AND R. J. ABBOTT. 2002. Molecular analysis of the Pleistocene history of*Saxifraga oppositifolia* in the Alps. *Molecular Ecology* 11: 1409–1418.
JAMES, T. Y., J. M. MONCALVO, S. LI, AND R. VILGALYS. 2001. Polymorphisms at the ribosomal DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. *Genetics* 157: 149–161.
LEGENDRE, P., AND L. LEGENDRE. 1998. Numerical ecology. Elsevier, Amsterdam, Netherlands.
MARDULYN, P. 2001. Phylogeography of the Vosges mountains populations of *Gonioctena pallida* (Coleptera: Chrysomelidae): a nested clade analysis of mitochondrial DNA haplotypes. *Molecular Ecology* 10: 1751–1763.
MASKAS, S. D., AND M. B. CRUZAN. 2000. Patterns of intraspecific diversification in the *Piriqueta caroliniana* complex in southeastern North America and the Bahamas. *Evolution* 54: 815–827.
MEUSEL, H., E. JÄGER, AND E. WEINERT. 1965. Vergleichende Chorologie der zentraleuropäischen Flora, Karten I. Fischer, Jena, Germany.
NORDAL, I., AND B. JONSELL. 1998. A phylogeographic analysis of *Viola rupestris*: three post-glacial immigration routes into the Nordic area? *Botanical Journal of the Linnean Society* 128: 105–122.
POSADA, D., K. A. CRANDALL, AND A. R. TEMPLETON. 2000. GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* 9: 487–488.
RICE, W. R. 1989. Analyzing tables of statistical tests. *Evolution* 43: 223–225.
ROHLF, F. J. 1997. NTSYS-PC. Numerical taxonomy and multivariate analysis system version 2.0. Applied Biostatistics, Setauket, New York, USA.
SEDDON, J. M., F. SANTUCCI, N. J. REEVE, AND G. M. HEWITT. 2001. DNA footprints of European hedgehogs, *Erinaceus europaeus* and *E. concolor*: Pleistocene refugia, postglacial expansion and colonization routes. *Molecular Ecology* 10: 2187–2198.
SPSS. 1999. SPSS. Version 9.0.0. SPSS, Chicago, Illinois, USA.
STEHLIK, I. 2000. Nunataks and peripheral refugia for alpine plants during Quaternary glaciation in the middle parts of the Alps. *Botanica Helvetica* 110: 25–30.
STEHLIK, I., F. R. BLATTNER, R. HOLDEREGGER, AND K. BACHMAN. 2002. Nunatak survival of the high alpine plant *Eritrichium nanum* (L.) Gaudin in the Central Alps during the ice ages. *Molecular Ecology* 11: 2027–2036.
STEHLIK, I., J. J. SCHNELLER, AND K. BACHMANN. 2001. Resistance or emigration: response of the high-alpine plant *Eritrichium nanum* (L.) Gaudin to the ice age within the Central Alps. *Molecular Ecology* 10: 357–370.
STEHLIK, I., J. J. SCHNELLER, AND K. BACHMANN. 2002. Immigration and *in situ* glacial survival of the low-alpine *Erinus alpinus* (Scrophulariaceae). *Biological Journal of the Linnean Society* 77: 87–103.
STEHLIK, I., A. TRIBSCH, AND P. SCHÖNSWETTER. 2001. First joint botanical mountain phylogeography meeting. *Bauhinia* 15: 69–90.
TEMPLETON, A. R. 1998. Nested clade analyses of phylogeographic data: testing hypothesis about gene flow and population history. *Molecular Ecology* 7: 381–397.
TEMPLETON, A. R., E. BOERWINKLE, AND C. F. SING. 1987. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics* 117: 343–351.
TEMPLETON, A. R., E. ROUTMAN, AND C. A. PHILLIPS. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* 140: 767–782.
TEMPLETON, A. R., AND C. F. SING. 1993. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics* 134: 659–669.
TREMBLAY, N. O., AND D. J. SCHOEN. 1999. Molecular phylogeography of *Dryas integrifolia*: glacial refugia and postglacial recolonization. *Molecular Ecology* 8: 1187–1198.
TURGEON, J., AND L. BERNATCHEZ. 2001. Mitochondrial DNA phylogeography of lake cisco (*Coregonus artedi*): evidence supporting extensive secondary contacts between two glacial races. *Molecular Ecology* 10: 987–1001.
VOS, P., R. HOGERS, M. BLEEKER, M. REIJANS, T. VAN DE LEE, M. HORNES, A. FRIJTES, J. POT, J. PELEMAN, M. KUIPER, AND M. ZABEAU. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407–4414.
WAGENITZ, G. 1981. Familie Polygonaceae. *In* G. Wagenitz [ed.], G. Hegi, Illustrierte Flora von Mitteleuropa, vol. III (1), 352–436. Parey, Berlin, Germany.
WIDMER, A., AND M. BALTISBERGER. 1999. Extensive intraspecific chloroplast DNA (cpDNA) variation in the alpine *Draba aizoides* L. (Brassicaceae): haplotype relationships and population structure. *Molecular Ecology* 8: 1405–1415.
ZHANG, L.-B., H. P. COMES, AND J. W. KADEREIT. 2001. Phylogeny and quaternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. *American Journal of Botany* 88: 2331–2345.