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Several Pleistocene refugia detected in the high alpine plant *Phyteuma globulariifolium* Sternb. & Hoppe (Campanulaceae) in the European Alps

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Abstract

***Phyteuma globulariifolium* is a high alpine plant species growing in the European Alps and the Pyrenees. In order to elucidate its glacial history, 325 individuals from 69 populations were analysed using the amplified fragment length polymorphism (AFLP) technique. A strongly hierarchical phylogeographical pattern was detected: Two major east–west vicariant groups can be separated along a gap in the distributional area. A further subdivision into at least four populational groups is in congruence with presumed peripheral glacial refugia. There is no indication for survival on unglaciated mountain tops (nunataks) in the interior of the Pleistocene ice shield covering the Alps. Our results favour glacial survival in peripheral, unglaciated or not fully glaciated areas. Populations of *P. globulariifolium* in the Pyrenees are the result of relatively recent long-distance dispersal. Within the Alps, there is strong differentiation among groups of populations, whereas within them the differentiation is weak. This suggests high levels of gene-flow over short to middle distances.**

Keywords: amplified fragment length polymorphism, European Alps, glacial refugia, phylogeography, Quaternary biogeography, recent long-distance dispersal

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Introduction

At the maximum extent of the last glaciation in Europe, large areas of the Alps were covered by a continuous ice-shield similar to the present situation in Greenland or Antarctica (Tallis 1991; Wilson *et al*. 2000). However, in the peripheral calcareous northeastern, southeastern and southwestern Alps, comparatively large areas remained unglaciated (Voges 1995; Fig. 1). They still harbour a remarkable number of endemic taxa confined to calcareous bedrock and thereby reveal their importance as peripheral refugial areas (Merxmüller 1952, 1953, 1954; Pawlowski 1970; Niklfeld 1972, 1973). For silicicolous plants, i.e. plants restricted to siliceous bedrock (approximately a third of the flora of the Alps), the situation is much less clear: they had far fewer opportunities to survive in peripheral refugia because the interior Alpine siliceous regions are usually flanked by limestone ranges. This is especially pronounced in the eastern Alps (Fig. 1). Only in parts of the western and the easternmost Alps, did large siliceous areas remain unglaciated or at least comprised territory below the Pleistocene snow line (Penck & Brückner 1909; Jäckli 1970; Van Husen 1987). These potential refugia (Fig. 1) show good congruence with distribution patterns of endemic or disjunctly distributed silicicolous taxa (A. Tribsch unpublished).

Additional to peripheral refugia, ice-free mountain tops ('nunataks') protruding above the glaciers are suspected to have harboured populations of higher plants and to have served as sources for postglacial recolonization. This 'nunatak' hypothesis and its counterpart, the 'tabula-rasa' hypothesis (i.e. total eradication of plant life and subsequent recolonization from refugia outside the ice-shield) have been debated since the early 20th century (reviewed in Brockmann-Jerosch & Brockmann-Jerosch 1926 and Stehlik *et al*. 2000 for the Alps and Dahl 1987; Nordal 1987; and Birks 1993 for Scandinavia). None of the molecular

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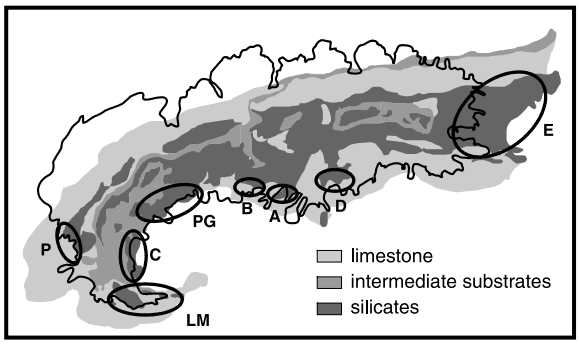

Fig. 1 Geology and Pleistocene glaciation of the European Alps. Information on geology is simplified from Schönenberg & Neugebauer (1994), the maximum extent of the Pleistocene ice shield as in Fig. 2. Presumed refugial areas for silicicolous vascular plants (ovals) are proposed based on geological and biological evidence (see text). A = southern Adamello; B = Alpi Bergamasche; C = Cottic Alps; D = southwestern Dolomites; EC = easternmost Austrian Central Alps; LM = Ligurian and Maritime Alps; P = Pelvoux; PG = southern Penninic and Grajic Alps.

investigations of Scandinavian plants, however, gave unambiguous evidence in favour of the nunatak hypothesis, and it thus has been concluded that 'glacial survival does not matter' (Brochmann *et al*. 1996; Gabrielsen *et al*. 1997; Tollefsrud *et al*. 1998). In the Alps, the debate has gained much interest recently due to application of new molecular techniques. Füchter *et al*. (2001) found high levels of chloroplast DNA (cpDNA) polymorphism in *Draba aizoides* in northern Alpine nunataks and in the central Alps suggesting survival on several nunataks. For *Eritrichum nanum*, nunatak survival is indicated based on chloroplast DNA-restriction fragment length polymorphism (cpDNA-RFLPs) and amplified fragment length polymorphism (AFLPs) (Stehlik *et al*. 2001, 2002ab). In contrast, nunatak survival could neither be proven nor falsified in *Saxifraga oppositifolia* in the Alps (Holderegger *et al*. 2002).

A model species to test the nunatak hypothesis should currently grow in the highest Alpine plant communities, to have been at least potentially able to cope with the hostile Pleistocene conditions on nunataks. Furthermore, its present distribution should not be restricted to formerly glaciated areas, allowing a direct comparison with populations in ice-free peripheral refugia. Among the ≈ 4500 vascular plant species in the Alps, only few, such as *Phyteuma globulariifolium*, *Ranunculus glacialis* or *Saxifraga bryoides* fulfil these criteria.

The aim of this study is to trace the glacial fate of the strictly silicicolous *Phyteuma globulariifolium* by applying the highly resolving AFLP technique (Vos *et al*. 1995) on 69 populations covering the entire distribution of the species. A major interest is which of the potential siliceous refugia (Fig. 1) at the margin of the Alps harboured populations of *P. globulariifolium* during the ice ages. Furthermore, we aim to differentiate periglacial survival from survival on nunataks. Nunatak survival, if not completely swamped by re-migrating genotypes (Gabrielsen *et al*. 1997; Tollefsrud *et al*. 1998; Holderegger *et al*. 2002), should lead to a patchy distribution of groups of related genotypes throughout the formerly glaciated central areas of the Alps, potentially differentiated and surrounded by peripheral genotypes. An example of this kind of glacial survival in the western central Alps is provided by Stehlik *et al*. (2001, 2002a). In contrast, immigration of refugial populations from peripheral refugia should result in large, relatively uniform areas populated by closely related genotypes without exhibiting much variation between populations. A reduction of genetic diversity in the course of postglacial remigration seems likely and has already been shown for other taxa (Broyles 1998; Shapcott 1998; Stehlik *et al*. 2002a,b; Tribsch *et al*. 2002; but see Abbott *et al*. 1995 for contrasting results).

Materials and Methods

The species

Phyteuma globulariifolium is endemic to the European Alps and the easternmost Pyrenees (Fig. 2). In the Alps it is widespread and occurs from the Ligurian Alps (Italy) to the easternmost high mountains in Styria (Austria) with some gaps in the distribution that do not have obvious ecological explanations (Welten & Sutter 1982; see Fig. 2). The species is a common element of stable, high alpine to subnival plant communities and is among the highest ascending vascular plants. In the Swiss Alps it is found up to 4010 m (Ellenberg 1996). The species also regularly descends below 2300 m and occurs in presumptive peripheral refugia in which higher summits are often lacking. Typical for the genus (Maier *et al*. 1999), seeds of the insect-pollinated proterandric *P. globulariifolium* are small, with no adaptation for long-distance dispersal. The same authors provided experimental and simulation data for seed dispersal in the closely related (Schulz 1904) alpine species *P. hemisphaericum*, concluding that only a small area around the mother plant is preferentially colonized and that long-distance dispersals are rare events.

P. globulariifolium s. l. has been split into two species (Schulz 1904): *P. pedemontanum* R. Schultz occurs in the western portion of the distributional area and *P. globulariifolium* in the east. In *Flora Europaea* (Damboldt 1976) this division, made at the subspecific level, was regarded as provisional, *P. pedemontanum* in particular needing further study. Hess *et al*. (1972) state that the differential characters used by Schulz (1904) do not effectively differentiate the taxa.

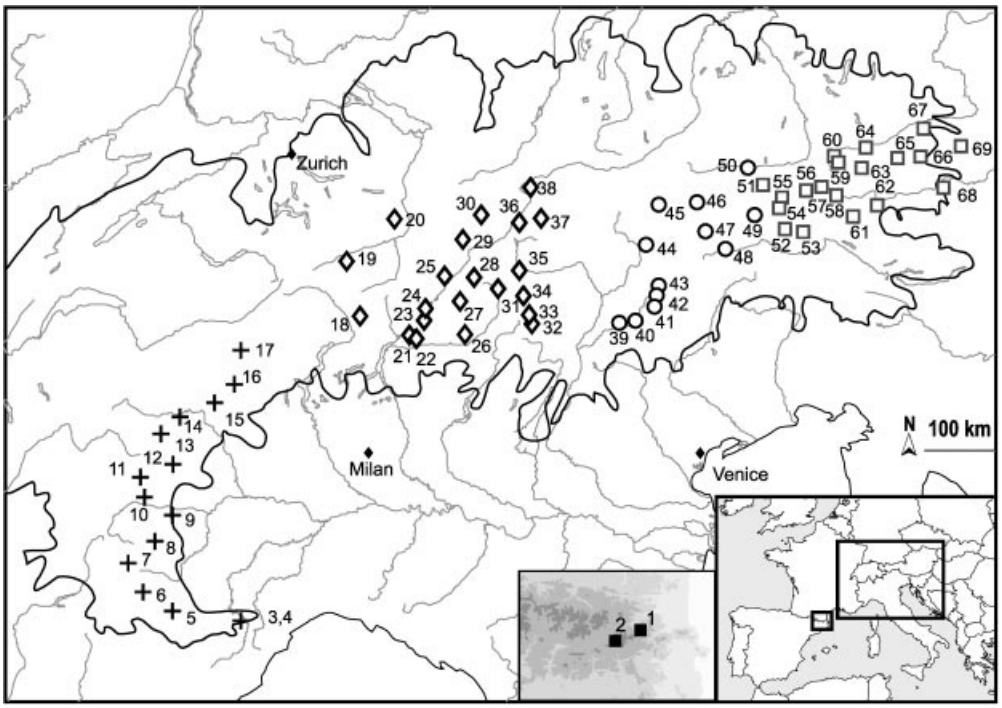

Fig. 2 Sampled populations of *Phyteuma globulariifolium* in the European Alps and in the Pyrenees along maximum extent of the Pleistocene ice shield during the last glacial period (black line; modified from Jäckli 1970; van Husen 1987; and Voges 1995). Bottom right insert indicates the position of the sampling areas in Europe: large rectangle = European Alps; small rectangle = eastern Pyrenees (magnified in the bottom left insert). Population numbers refer to Table 1. The sampling is comprehensive, gaps reflect natural breaks in distribution. (■) Pyrenean populations of W1, (+) Alpine populations of W1; (◇) W2; (○) E1; (□) E2.

Sampling

Sixty-nine populations of *P. globulariifolium* (Table 1) were sampled throughout its distribution in the Alps and the Pyrenees. In most cases, leaf material of five plants per population was collected. Exceptions are populations 39 and 64 with two plants; 57, 59 and 61 with three; and 1, 2, 21, 36, 42, 45, 51 and 56 with four. Voucher specimens of all populations are deposited in the herbarium of the Institute of Botany of the University of Vienna (WU). Following Taberlet *et al*. (1998), special emphasis was given to potential refugia, in our case putative peripheral refugia.

DNA isolation and AFLP fingerprinting

Young shoots (flowers and buds were removed) were collected in the field and immediately stored in silica gel. Total genomic DNA was extracted following the CTAB protocol (Doyle & Doyle 1987) with the following modifications: after precipitation with isopropanol and subsequent centrifugation, the DNA pellet was washed with 70% ethanol, dried at 37 °C and resuspended in TE buffer. The quality of the extracted DNA was checked on 1% TAE-agarose gels. The amount of DNA was estimated photometrically (UV-160 A, Shimadzu).

Genomic DNA (≈ 500 ng) was digested with *Mse*I (New England BioLabs) and *Eco*RI (Promega), ligated (T4-ligase; Promega) to double-stranded adapters and pre-amplified using the AFLP Ligation and Preselective Amplification Module (PE Applied Biosystems) for regular genomes following the manufacturer's instructions (PE Applied Biosystems 1996). Incubation of the restriction–ligation reactions (2 h at 37 °C) as well as polymerase chain reactions (PCR) were performed on a GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems). Deviating from the original protocol, PCRs were run in a reaction volume of 5 μL. An initial screening using nine selective primer combinations was performed on three individuals from two populations using the Selective Amplification Start-up Module for Regular Genomes (PE Applied Biosystems). Three primer combinations giving clear and reproducible bands and showing variation within and between populations were chosen for further analysis (*Eco*RI ACA-*Mse*I CAT; *Eco*RI AAG-*Mse*I CTG; *Eco*RI AAC-*Mse*I CTT). On several samples, independent AFLP reactions were performed for internal control. The fluorescence-labelled selective amplification products were separated on a 5% polyacrylamide gel with an internal size standard (GeneScan®-500 [ROX], PE Applied Biosystems) on an automated sequencer (ABI 377, Perkin–Elmer). Raw data were collected

Table 1 Numbering of populations, location, country (A = Austria; CH = Switzerland; E = Spain; F = France; I = Italy); population group, coordinates, number of fragments (Frag.) per population; percentage of fragments which exhibit intrapopulational polymorphism ($\%_{POLY}$); Shannon diversity index (H_{Sh}); number of private fragments (N_P) and average of rare (< 30 occurrences in the data set) fragments per individual (N_{RI}) of the 69 investigated populations of *Phyteuma globulariifolium*. The genetic diversity measures $\%_{POLY}$ and H_{Sh} are only given for populations with four or five nonidentical individuals

Population	Location	Country	Popn group	Coordinates (E/N)	Frag.	$\%_{POLY}$	H_{Sh}	N_P	N_{RI}
1	Canigou	F	W1	2.43°/42.51°	68	—	—	1	3.25
2	Puigmal	F/E	W1	2.12°/42.38°	80	37.5	8.83		4.25
3	Bocchetta Aseo	I	W1	7.78°/44.15°	91	42.9	12.28	3	5.4
4	Cima delle Roccate	I	W1	7.78°/44.15°	92	44.6	12.86	1	5.0
5	Col Lombard	F/I	W1	7.15°/44.20°	97	48.5	14.47	3	6.0
6	Col Restefond	F/I	W1	6.85°/44.34°	95	47.4	13.52	1	8.0
7	Col de Var	F/I	W1	6.70°/44.54°	95	43.2	12.01	2	8.4
8	Col Agnel	F/I	W1	6.98°/44.68°	91	41.8	11.40	1	6.4
9	Punta Cialancia	I	W1	7.12°/44.88°	93	49.5	13.15	2	5.0
10	Monte Genevris	I	W1	6.88°/45.00°	84	39.3	9.59		3.8
11	Col Sommelier	I	W1	6.83°/45.12°	87	42.5	10.80	2	3.6
12	Monte Palon	I	W1	7.14°/45.21°	93	43.0	12.32	2	4.2
13	Col de l'Iseran	F/I	W1	7.02°/45.42°	93	48.4	13.23	3	3.8
14	Gran Paradiso	I	W1	7.20°/45.53°	94	56.4	15.49	1	5.6
15	Champorcher	I	W1	7.55°/45.62°	99	54.5	16.40	1	5.0
16	Monte Nery	I	W1	7.74°/45.77°	87	46.0	11.69		2.2
17	Gornergrat	CH	W1	7.80°/45.98°	85	44.7	11.76		3.6
18	Cima dell'Uomo	CH	W2	8.95°/46.23°	82	43.9	9.65		2.2
19	Passo Lucomagno	CH	W2	8.80°/46.58°	89	41.6	11.46	1	3.0
20	Cassonsgrat	CH	W2	9.27°/46.87°	90	44.4	11.55	1	2.4
21	Monte Legnone	I	W2	9.42°/46.10°	88	42.0	11.38		1.25
22	Monte Rotondo	I	W2	9.49°/46.07°	74	—	—		0.0
23	Monte Spluga	I	W2	9.55°/46.18°	83	44.6	10.70		1.0
24	Passo d'Oro	I	W2	9.57°/46.26°	85	37.6	9.74		2.0
25	Piz Julier	CH	W2	9.75°/46.48°	82	43.9	10.61		1.2
26	Bocchetta Stefano	I	W2	9.96°/46.11°	86	27.9	7.47		4.4
27	Bocchetta Forbici	I	W2	9.90°/46.32°	84	47.6	11.81		0.4
28	Monte Breva	I	W2	10.05°/46.47°	94	48.9	14.07	3	2.0
29	Schwarzhorn	CH	W2	9.93°/46.73°	86	40.7	10.46		0.6
30	Hohes Rad	A	W2	10.10°/46.88°	77	33.8	7.21		0.0
31	Cima Piazzi	I	W2	10.27°/46.41°	95	52.6	15.11	1	2.6
32	Val Folgorida	I	W2	10.61°/46.17°	84	35.7	9.12		0.8
33	Passo Paradiso	I	W2	10.57°/46.23°	88	45.5	11.82		1.4
34	Passo di Gavia	I	W2	10.47°/46.33°	84	47.6	11.09		1.2
35	Monte Torraccia	I	W2	10.43°/46.52°	88	46.6	11.85		2.4
36	Piz Lad	CH/I	W2	10.47°/46.83°	77	32.5	7.23		0.25
37	Weißseejoch	A	W2	10.68°/46.87°	79	34.2	7.47		0.0
38	Sattelkopf	A	W2	10.58°/47.08°	78	37.2	7.91		0.2
39	Monte Ziolera	I	E1	11.45°/46.17°	81	—	—		4.5
40	Cima d'Asta	I	E1	11.60°/46.18°	82	23.2	5.33		3.4
41	Cavalazza Piccola	I	E1	11.79°/46.30°	78	23.1	5.40	2	3.4
42	Cima Margerita	I	E1	11.80°/46.38°	85	32.9	8.00		3.75
43	Passo Pordoi	I	E1	11.82°/46.42°	81	22.2	4.75		3.2
44	Plose	I	E1	11.71°/46.68°	80	20.0	4.21		3.8
45	Tristenspitz	I	E1	12.18°/46.97°	83	25.3	6.36		2.75
46	Totenkarspitze	A	E1	12.19°/46.98°	90	41.1	10.58		2.4
47	Pfannhorn	A	E1	12.28°/46.78°	85	30.6	7.11		1.4
48	Col Quaterna	I	E1	12.47°/46.67°	84	35.7	8.10		1.8
49	Schleinitz	A	E1	12.75°/46.90°	89	33.7	8.92		3.0
50	Kitzsteinhorn	A	E1	12.68°/47.20°	82	34.1	7.32		0.8
51	Hochtor	A	E2	12.83°/47.08°	90	38.9	10.82		1.0
52	Gerberhütte	A	E2	13.03°/46.82°	81	34.6	8.27		0.6

Table 1 *Continued*

Population	Location	Country	Popn group	Coordinates (E/N)	Frag.	$\%_{POLY}$	H_{Sh}	N_P	N_{RI}
53	Scharnik	A	E2	13.04°/46.80°	76	28.9	6.01		0.2
54	Sadnig	A	E2	12.98°/46.93°	84	45.2	10.43		0.8
55	Wurtenkees	A	E2	13.02°/47.02°	87	46.0	11.85		1.4
56	Ankogel	A	E2	13.25°/47.05°	82	34.1	8.13		1.75
57	Hafner	A	E2	13.40°/47.07°	83	—	—		3.33
58	Wandspitze	A	E2	13.53°/47.02°	88	47.7	12.33	1	2.8
59	Gamsleitenspitz	A	E2	13.55°/47.24°	86	—	—		1.67
60	Seekarspitz	A	E2	13.55°/47.28°	89	40.4	10.33		1.6
61	Rosenock	A	E2	13.72°/46.88°	80	—	—	1	1.67
62	Bretthöhe	A	E2	13.93°/46.91°	82	42.7	10.15	2	1.6
63	Gumma	A	E2	13.78°/47.21°	87	39.1	10.00		1.2
64	Hochwildstelle	A	E2	13.83°/47.34°	77	—	—		1.5
65	Aarfeldspitz	A	E2	14.09°/47.27°	82	40.2	9.96	1	1.0
66	Schießeck	A	E2	14.33°/47.24°	78	35.9	8.22	1	1.0
67	Dreistecken	A	E2	14.40°/47.46°	84	41.7	10.14		0.8
68	Zirbitzkogel	A	E2	14.57°/47.07°	86	39.5	9.97	2	2.4
69	Lamprechtshöhe	A	E2	14.78°/47.34°	91	40.7	10.88	2	3.2

and aligned with the internal size standard using the ABI Prism GeneScan® Analysis Software (PE Applied Biosystems). Subsequently, the GeneScan files were imported into GENOGRAPHER (version 1.1.0, © Montana State University 1998; http://hordeum.msu.montana.edu/genographer/) to score the fragments. Each AFLP fragment was scored using the 'thumbnail' option of the program, which allows comparison of the signal per locus over all samples. AFLP fragments that exhibited ambiguous peaks were excluded from the analysis. Peaks of low intensity were included into the analysis when unambiguous scoring was possible. The results of the scoring were exported as a presence/absence matrix and used for further manipulation.

Data analysis

Shannon siversity $H_{Sh} = -\Sigma(p_j \ln p_j)$, in which p_j is the relative frequency of the j-th fragment (Legendre & Legendre 1998), the number of AFLP fragments per population and the percentage ($\%_{POLY}$) of fragments which are polymorphic within each population, were calculated for all populations. Boxplot diagrams of H_{Sh} of population groups were calculated and plotted with SPSS 8.0.0 (SPSS Inc. 1989–97). As additional diversity marker, the mean number of rare fragments per individual (N_{RI}), and the number of private (N_P) and fixed private fragments per population was estimated. Fragments were treated as rare when they occurred in fewer than 30 of the 325 investigated individuals. Private fragments are confined to a single population and fixed private fragments are found in all investigated individuals of a single population.

A neighbour-joining analysis based on pairwise F_{ST} (Φ_{ST}) comparisons between populations (ARLEQUIN Version 1.1; Schneider *et al*. 1997) was computed in order to search for groupings of populations with NTSYS-PC Version 2.0 (Rohlf 1997). A principal coordinate analysis (PCoA) based on a matrix of between-individual Jaccard similarities ($C_J = a/a + b + c$, where a is the number of fragments shared between two individuals and b and c are present in only one individual), was calculated and plotted with SPSS Version 8.0.0. Analyses of molecular variance (AMOVAS), which partition the overall genetic variation into levels (within populations, among populations, eventually among groups of populations), were calculated with ARLEQUIN Version 1.1 (Schneider *et al*. 1997).

Mantel tests were applied in two different ways: (i) to compare the genetic matrix of interindividual Jaccard distances ($D = 1 - C_J$) with a matrix of geographical distances in km; and (ii) to test the goodness-of-fit of the genetic distance matrix with a model matrix of distance-classes (Gabrielsen *et al*. 1997) where the km-distances between individuals were coded into 15 classes. All Mantel R_M-values were calculated and Bonferroni-corrected using the R-PACKAGE Version 4.0 (Casgrain & Legendre 1999).

Results

AFLP-patterns and polymorphism; intrapopulational genetic diversity

With the three primer combinations used, 257 unambiguously scoreable fragments have been generated of which 240 (93.4%) are polymorphic. The length of the fragments

varies from 48 to 472 bp. Identical genotypes were detected in populations 1 (three individuals), 8 (two individuals), 13 (two individuals), 22 (three individuals), 24 (two individuals), 26 (two individuals) and 32 (two individuals).

The number of fragments per population varies from 68 in population 1 to 99 in population 15 (mean = 85.36, SD = 5.93; Table 1). The percentage of fragments which are polymorphic within a certain population varies from 20.0% in population 44 to 56.4% in population 14 (mean $\%_{POLY}$ = 39.94, SD = 7.87; Table 1). H_{Sh} ranges from 4.2 in population 44 to 16.4 in population 15 (mean H_{Sh} = 10.18, SD = 2.65; for both diversity indices only populations with four or five nonidentical individuals were taken into account; Table 1). Both diversity measures are significantly correlated (Pearson 2-tailed, P = 0.01). Private fragments (N_p; 1–3 per population) were found in 25 populations (Table 1). A single fixed private fragment exists in population 6. The mean number of rare fragments (N_{RI}) varies from zero in populations 22, 30 and 37 to 8.4 in population 7 (mean N_{RI} = 2.56, SD = 1.87; Table 1).

Clustering of populations and geographical structure

Neighbour-joining analysis of populations (Fig. 3) revealed a substantial separation into a western group comprising the Pyrenees and the western Alps (from here on referred to as W) and an eastern group (E). W and E are characterized by two fixed private fragments each, and by 110 and 43 private fragments, respectively. In the 186 individuals of W containing a total number of 197 fragments, 21 fragments are fixed. A total of 130 fragments are found among the 139 individuals from E, of which 28 fragments are fixed. Both W and E can be further subdivided. W is split into W1 (58 individuals) including the populations from the Pyrenees and the western part of the Alps and W2 (128 individuals) comprising the middle part of the Alps from Furka Pass in Switzerland to the Etsch Valley in Italy and Brenner Pass at the Austrian/Italian border. E is subdivided into a western (E1; 55 individuals) and an eastern (E2; 84 individuals) unit within the Hohe Tauern chain east of Mt. Grossglockner (Austria). All of the proposed groups have private fragments: 35 are found in W1, 13 in W2, seven in E1 and 12 in E2. Only 35 fragments are found in all population groups.

PCoA (Fig. 4) confirmed the regional pattern detected by the neighbour-joining analysis: W and E are separated with a large percentage of the variation explained by the first factor (24.4%). W1 and W2 are well differentiated, the Pyrenean populations cluster with W1. Along the second factor (19.3%), regions E1 and E2 form well-separated entities. The third factor (11.8%) provides no additional information, as the clouds of E1 and E2 only change place. PCoAs calculated for the eastern or western groups separately (data not shown) lead to congruent results.

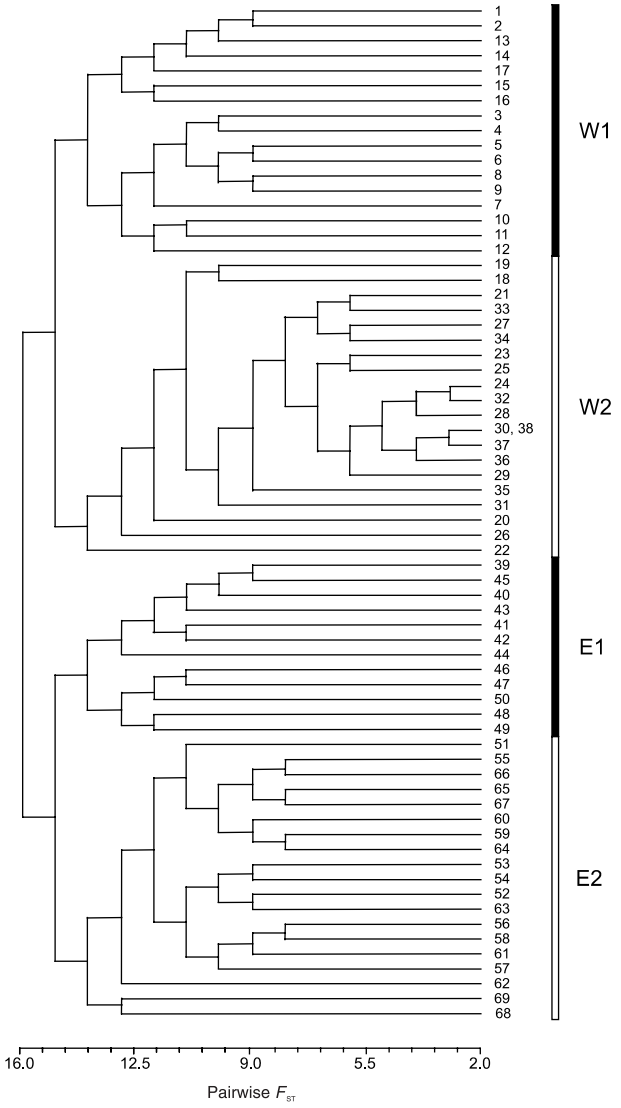

Fig. 3 Neighbour-joining analysis of 69 populations of *Phyteuma globulariifolium* based on pairwise F_{ST} (Φ_{ST}) values.

AMOVAS, Mantel tests, comparison of H_{Sh} *among groups*

AMOVAS confirm the clear phylogeographical structure in *Phyteuma globulariifolium* (Table 2). If only two levels of variation (i.e. within and among populations) are introduced, 58.5% of the overall variation account for variation among populations. If a third level (variation among the four above-defined groups of populations) is added, more than one-half (51.2%) of the overall variation is attributed to variation among groups, 36.2% to variation within populations and only 12.6% to that between populations within groups. When differentiating E and W only, 49.3% of the overall genetic variation accounts for variation between them. However, within W, 22.5% can be attributed to variation between W1 and W2; within E, the subregional differentiation between E1 and E2 is even more with 38.1%.

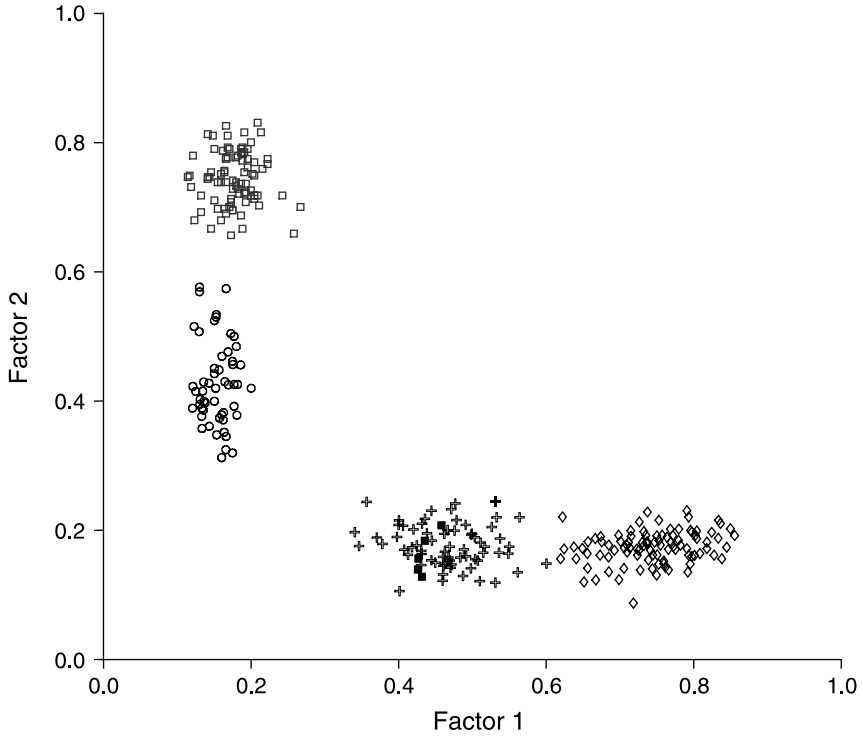

Fig. 4 Principal coordinate analysis (PCoA; first two factors) of all investigated individuals of *Phyteuma globulariifolium*. Symbols as in Fig. 2.

Table 2 Analysis of molecular variance (AMOVA) for amplified fragment length polymorphism (AFLP) phenotypes in *Phyteuma globulariifolium*

Source of variation	d.f.	Sum of squares	Variance components	% Total variance	F_{ST}*
Among populations (total)	68	4346.55	11.80	58.50	0.59
Within populations	256	2142.32	8.37	41.50	
Among W1, W2, E1, E2	3	2909.89	11.84	51.19	0.64
Among populations	65	1436.65	2.92	12.62	
Within populations	256	2142.32	8.37	36.19	
Among W1 and W2	1	362.70	3.66	22.49	0.44
Among populations	36	947.18	3.52	21.63	
Within populations	148	1345.40	9.10	55.88	
Among E1 and E2	1	409.75	5.9	38.31	0.52
Among populations	29	489.48	2.13	13.79	
Within populations	108	796.92	7.38	47.89	

*All *P*-values were < 0.001.

The overall R_M-value calculated with genetic and geographical distance matrices is 0.188 ($P = 0.001$), but it rises to 0.679 ($P = 0.001$) when populations from the Pyrenees (1 and 2) are excluded from the analysis. Genetic distances significantly increase with geographical distances therefore, but the correlation is much stronger if only Alpine populations are considered. The Mantel test with distance classes (Fig. 5) reveals a significantly positive correlation among populations separated by up to 140 km. The correlation is significantly negative from 140 to 700 km distance. The highest R_M-value was found among populations 40–60 km apart ($R_M = 0.32$, $P = 0.001$), the lowest among populations isolated by 500–700 km, i.e. the maximal distances within the distribution of *P. globulariifolium* in the Alps ($R_M = -0.29$, $P = 0.001$). In distance classes that only occur between the Pyrenean populations and those from the Alps (distance classes 14 and 15), the R_M-value again increases to zero (not significant) and −0.12 ($P = 0.001$), respectively.

H_{Sh} varies strongly among the four geographical regions. The only nonsignificant correlations (Mann–Whitney *U*-test, $P < 0.005$) exist between W1 and W2 and W2 and E2, respectively (Fig. 6).

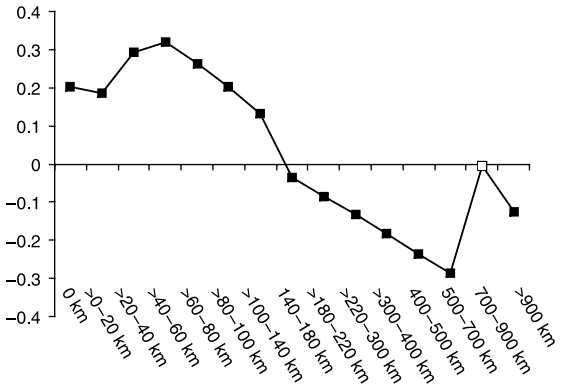

Fig. 5 Correlogram of Mantel R_M per distance class. Filled squares indicate Bonferroni-corrected R_M-values significantly different from 0 at $P \leq 0.05$.

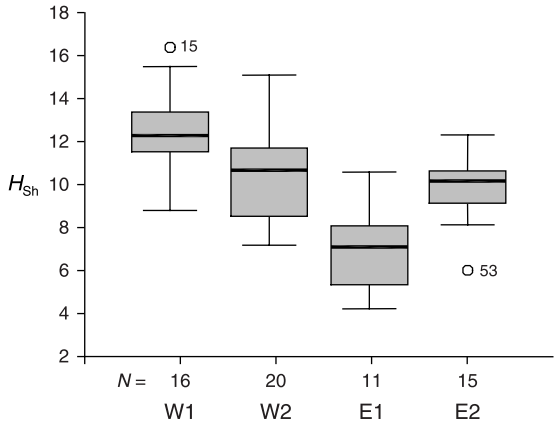

Fig. 6 Boxplots of H_{Sh} (Table 1) for the regional population groups W1, W2, E1 and E2 of all investigated individuals of *Phyteuma globulariifolium*. Outliers are labelled with the population number.

Discussion

The hierarchical structure of genetically defined groups of populations in the phylogeography of *Phyteuma globulariifolium* suggests at least two time horizons: (i) an ancient strong differentiation between E and W; and (ii) a younger structure within these two regions, possibly resulting from the last (Würm) glaciation.

Two ancient groups

Strong genetic differentiation between E and W populations contrasts with the poorly resolved morphological and taxonomic status of the vicariant taxa *P. globulariifolium* (east) and *'P. pedemontanum'* (west). E and W genetic patterns, however, are largely congruent with the presumptive distribution of the two taxa as recognized by Schulz (1904). The presence of several fixed private AFLP fragments is best interpreted as evidence for long-term isolation and restriction of gene flow between the groups. There is no indication of a hybrid zone, and furthermore, contact between E and W is currently not probable because these areas are separated by a distribution gap of ≈ 50 km (Fig. 2). Southward, this gap is formed by the Etsch Valley and adjacent limestone ranges, being hostile environments for *P. globulariifolium*. Northward, no ecological or geomorphological barrier exists in large mountainous high siliceous areas. In contrast to the unclear taxonomical situation suggested in floras (Hess *et al.* 1972; Damboldt 1976) therefore we face an ancient, but cryptic, evolutionary split through the Alpine distribution of *P. globulariifolium*. Congruent with AFLP data, sequences of the *trn*L-F-region of cpDNA revealed considerable differences between E and W (7 nucleotide substitutions of 935 bp present in all 13 accessions), whereas only single nucleotide changes exist within groups (G. M. Schneeweiss unpublished). The pattern detected in *P. globulariifolium* parallels that found in many species pairs, as east–west vicariance is relatively frequent in the flora of the Alps (Pawlowski 1970; Ozenda 1988).

Glacial refugia

The phylogeographical pattern detected in *P. globulariifolium* suggests the existence of (at least) four glacial refugia in the Alps that gave rise to the genetically defined regional population groups. The subdivisions suggested by neighbour-joining analysis and PCoA (Figs 3 and 4) are corroborated by the existence of a high number of private fragments in each of the population groups W1, W2, E1 and E2. Within these groups, a low percentage of variation between populations (Table 2) indicates their close relationship. The relatively strong genetic differentiation between E1 and E2 argues against a continuous geographical distribution within *P. globulariifolium*. All other genetic groups have a strong geographical component. Between E1 and E2, however, there is evidence for secondary introgression indicated by 15 fragments shared between the easternmost populations of E1 (populations 46–50) and E2.

The population groups show good congruence with the proposed refugia for silicicolous plants (Fig. 1). Their existence is indicated by geological data (Penck & Brückner 1909; Jäckli 1970; van Husen 1987); they contained unglaciated siliceous bedrock (W1, W2, E2) or at least unglaciated siliceous territory below the snowline within the ice-shield (E1). Furthermore, most of these regions are also characterized by the occurrence of endemic silicicolous plants (Pawlowski 1970). W1 overlaps with three presumed refugia in the Ligurian to Maritime Alps, Cottic Alps, and Grajic to Penninic Alps. W2 spans two refugia, i.e. western Alpi Bergamasche and southern Adamello. E1 can be

related to the southwestern Dolomites, whereas E2 covers the refugium in the easternmost Central Alps. The proposed refugium Pelvoux is not pertinent for this study, because *P. globulariifolium* does not occur there.

Recent long-distance dispersal

The few populations of *P. globulariifolium* in the Eastern Pyrenees (populations 1 and 2) appear to be the result of a relatively recent colonization event via long-distance dispersal from W1 (perhaps with a stepping stone in the Massif Central in France, which no longer exists due to the lack of high Alpine habitats). This is suggested by both the species distribution pattern (restriction to parts of the Pyrenees that are closest to the alps) and the molecular data. The two populations are nested within W1 (Figs 3 and 4), and the most closely related populations are those from the Grajic (populations 13–15) and southern Penninic Alps (populations 16 and 17). Although providing clear evidence for relatively recent long-distance dispersal is difficult (Cain *et al*. 2000), evidence is accumulating that this phenomenon is not rare, especially in Arctic and Alpine taxa (Brochmann *et al*. 1996; Aares *et al*. 2000; Hagen *et al*. 2001; Tribsch *et al*. 2002).

Evidence for nunatak survival in central parts of the Alps?

The persistence of vascular plants on ice-free mountain tops protruding above Pleistocene glaciers (nunataks) has remained controversial (see Introduction). To facilitate the discussion, we offer the following perspectives. Geological evidence shows that there have been nunataks throughout the Alpine ice-shield (e.g. van Husen 1987), and it is reasonable therefore to distinguish between those in the centre (here referred to as 'central nunataks') and those close to the margin ('peripheral nunataks'). The latter have been neglected in the biogeographical literature, but obviously they offered more favourable climatic conditions.

The clear phylogeographical pattern within *P. globulariifolium* allows comment on the nunatak discussion. The low level of genetic population differentiation within W1, W2, E1 and E2, all of which include formerly unglaciated or not fully glaciated areas (Fig. 1), can be regarded as evidence against survival on central nunataks. Such survival in addition to survival in peripheral refugia would have introduced higher levels of population differentiation within groups. Our data showing no indication of genetic depauperation preferentially suggest survival of large populations in peripheral refugia that were isolated by glacier tongues and limestone ranges.

The distribution of genetic diversity measured with Shannon diversity index (H_{Sh}) shows no clear correlation with Pleistocene glaciations, as some peripheral populations (e.g. 3, 4, 62, 66–69) have a lower H_{Sh} than more interior ones (Table 1). The same pattern is observed within E2 where populations 62 and 66–69 are less diverse than more interior ones. Instead of attempting correlation of genetic diversity with the two classes of formerly glaciated and unglaciated areas, we can compare the size of the respective refugial area (and therefore source of postglacial spread) of the geographical subunits. W1 has a significantly higher and E1 a significantly lower level of H_{Sh} (Fig. 6). The distribution of rare and private fragments (Table 1) shows a parallel pattern. Geological evidence favours a large peripheral refugium for W1 (Voges 1995), whereas most probably only a small peripheral nunatak area surrounded by ice and limestone was available for the source populations of E1 (Fig. 1). Thus, population bottlenecks in E1 and large and diversified population survival in W1 might explain this pattern. During re-immigration from these source populations after the ice receded, much of the remnant genetic diversity was conserved (maybe due to leptokurtic dispersal and lack of 'leading edge'-migration; Hewitt 1996). Support for this hypothesis is provided by the low number of private fragments and the almost complete lack of fixed private alleles of populations in these refugia. This concords with results of the Mantel test with distance classes (Fig. 5) revealing a high similarity between individuals up to a distance of 100–140 km. True long-distance gene flow obviously is not frequent enough to obliterate the clear phylogeographical structure in *P. globulariifolium*. An additional indication for this is given by the ecologically unexplainable gaps in the distribution of the species. In contrast, simulation experiments (Maier *et al*. 1999) in the closely related *P. hemisphaericum* suggest that dispersals even over medium distances are rare events.

Conclusions

Studies like ours, based on extensive sampling of individual species, cannot explain the glacial survival of an entire biota in particular regions. They can contribute, however, to a comparative phylogeographical approach as advocated by, for example, Bermingham & Moritz (1998) and Hewitt (2001). They can also provide new insights into the influences of climatic fluctuations on the evolution and distribution of biodiversity. The different phylogeographical patterns exhibited by plants of similar altitudinal distribution such as *Eritrichum nanum* (Stehlik *et al*. 2001, 2002a) and *Phyteuma globulariifolium*, indicate that predictions of modes of glacial survival based on recent ecological conditions will be inadequate. The lack of evidence for survival on central nunataks in *P. globulariifolium* need not, therefore, contradict the results of Stehlik *et al*. (2001, 2002a,b) which support such as possibility. We are still far from a conclusive understanding of the complex processes that led to the genetic and organismic patterns of biodiversity in the Alps.

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This work is part of a comparative phylogeographical project investigating the response of 10 vascular plant taxa of the European Alps with contrasting distribution patterns and ecological requirements to Quaternary glaciations. P. Schönswetter is working with five high alpine to subnival species; A. Tribsch with five low alpine species. M. Barfuss is diploma student investigating the phylogeny of Bromeliaceae. H. Niklfeld's interests focus on comparative interpretation of distributional patterns of vascular plants in Central Europe.
