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Phylogeography of *Pulsatilla vernalis* (L.) Mill. (Ranunculaceae): chloroplast DNA reveals two evolutionary lineages across central Europe and Scandinavia

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ABSTRACT

Aim The aim of this study was to test hypotheses regarding some of the main phylogeographical patterns proposed for European plants, in particular the locations of glacial refugia, the post-glacial colonization routes, and genetic affinities between southern (alpine) and northern (boreal) populations.

Location The mountains of Europe (Alps, Balkans, Carpathians, Central Massif, Pyrenees, Scandinavian chain, Sudetes), and central European/southern Scandinavian lowlands.

Methods As our model system we used *Pulsatilla vernalis*, a widely distributed European herbaceous plant occurring both in the high-mountain environments of the Alps and other European ranges and in lowlands north of these ranges up to Scandinavia. Based on a distribution-wide sampling of 61 populations, we estimated chloroplast DNA (cpDNA) variation along six regions using polymerase chain reaction–restriction fragment-length polymorphisms (PCR–RFLPs) (*trn*H–*trn*K, *trn*K–*trn*K, *trn*C–*trn*D, *psb*C–*trn*S, *psa*A–*trn*S, *trn*L–*trn*F) and further sequencing of *trn*L–*trn*F and *trn*H–*psb*A. In addition, 11 samples of other European species of *Pulsatilla* were sequenced to survey the genus-scale cpDNA variation.

Results Eleven PCR–RFLP polymorphisms were detected in *P. vernalis*, revealing seven haplotypes. They formed two distinct genetic groups. Three haplotypes representing both groups dominated and were widely distributed across Europe, whereas the others were restricted to localized regions (central Alps, Tatras/Sudetes mountains) or single populations. Sequencing analysis confirmed the reliability of PCR–RFLPs and homology of haplotypes across their distribution. The chloroplast DNA variation across the section *Pulsatilla* was low, but *P. vernalis* did not share haplotypes with other species.

Main conclusions The genetic distinctiveness of *P. vernalis* populations from the south-western Alps with respect to other Alpine populations, as well as the affinities between the former populations and those from the eastern Pyrenees, is demonstrated, thus providing support for the conclusions of previous studies. Glacial refugia in the Dolomites are also suggested. Isolation is inferred for the high-mountain populations from the Tatras and Sudetes; this is in contrast to the case for the Balkans, which harboured the common haplotype. Specific microsatellite variation indicates the occurrence of periglacial lowland refugia north of the Alps, acting as a source for the post-glacial colonization of Scandinavia. The presence of different fixed haplotypes in eastern and western Scandinavia, however, suggests independent post-glacial colonization of these two areas, with possible founder effects.

Keywords **Alpine, boreal, Europe, northern refugia, phylogeography, post-glacial migrations, Scandinavia.**

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INTRODUCTION

Quaternary climatic fluctuations have strongly influenced the flora of central and northern Europe and have remodelled the distribution of plant taxa (Comes & Kadereit, 1998; Taberlet *et al.*, 1998). The present distributions of the majority of European plants species have been influenced or directly shaped by post-glacial migrations from refugia, where these species survived during periods of glaciation. Analyses of spatial genetic patterns in contemporary populations have been useful in inferring post-glacial histories of a number of species and in identifying refugia and migration routes. Although every species has its own specific phylogeographical history (Taberlet *et al.*, 1998), groups of species with similar ecological demands may have been similarly affected by glaciations, as in the case of silicicolous high-alpine species in the Alps (Schönswetter *et al.*, 2004a, 2005). Several large phylogeographical data sets have been produced during the last decade, but they focused mainly on European tree species for which good palaeobotanical data are available (e.g. Konnert & Bergmann, 1995; Demesure *et al.*, 1996; King & Ferris, 1998; Petit *et al.*, 2002; Grivet & Petit, 2003; Palmé *et al.*, 2003; Heuertz *et al.*, 2004), and on plant species of high mountains (Schönswetter *et al.*, 2005; and references therein) or northern latitudes (Abbott & Brochmann, 2003; Brochmann *et al.*, 2003; and references therein). In order to obtain a comprehensive picture of post-glacial recolonization processes in the European flora, it may be particularly useful to study species with various ecological niches and spanning different distributional elements. So far, there are few European-wide studies of widely distributed herbaceous plants or dwarf shrubs. Rendell & Ennos (2002) studied cpDNA variability in the European populations of *Calluna vulgaris* and discussed possible last glacial refugia for this common species. An amplified fragment length polymorphism (AFLP) study on *Trollius europaeus* spanning its European distribution, although based on a limited sampling, inferred possible sources for the post-glacial recolonization of Scandinavia (Després *et al.*, 2002). Post-glacial migration routes in Eurasia, aimed at determining the history of the central European populations, were also analysed for *Arabidopsis thaliana* (Sharbel *et al.*, 2000). However, despite some other notable exceptions (e.g. Alsos *et al.*, 2005), extensive intraspecific studies based on a large number of European populations are still scarce.

In the present paper, we analyse chloroplast DNA (cpDNA) variation in *Pulsatilla vernalis*. This species can be considered as a model for European plants that occur both in mountains and in lowlands (Pawłowski, 1928). The distribution of *P. vernalis* is limited to Europe. Its range spans most of the European alpine system (Ozenda, 1985), including subalpine and alpine habitats of the Pyrenees, the Alps, the Carpathians, the Sudetes and the Balkans, as well as the highest massifs of the Scandinavian chain in Norway. The species also occurs in the lowland areas of central Europe (mainly Germany and Poland), and in southern Scandinavia (Norway, Sweden, Denmark and Finland) (Meusel *et al.*, 1965; Fig. 1). We infer the phylogeography of *P. vernalis* in order to draw conclusions about some of the main phylogeographical patterns that have been proposed for European plants, in particular the location of glacial refugia, the post-glacial colonization routes, and the patterns of genetic affinities between southern (alpine) and northern (boreal) populations. Within this framework, we ask the following specific questions: (1) Is there a congruence of spatial genetic patterns in the Alps and other parts of the European alpine system between species with wider ecologically variable distributions and obligate high-mountain species, thus indicating a common set of refuges and migration routes? (2) Were northern refugia (on the plains north of the Alps) possible for high-mountain taxa not restricted to the alpine zone? (3) Where are the historical refugia for lowland parts of the distribution of high-mountain plants in central Europe and southern Scandinavia?

MATERIALS AND METHODS

The study species

Pulsatilla vernalis (L.) Mill. (Ranunculaceae) is an early spring-flowering hemicryptophyte 3–20 cm in height. Its thick rhizome shows occasional vegetative propagation. In the mountains the species occurs mostly on siliceous bedrock in dry subalpine shrub communities and alpine grasslands,

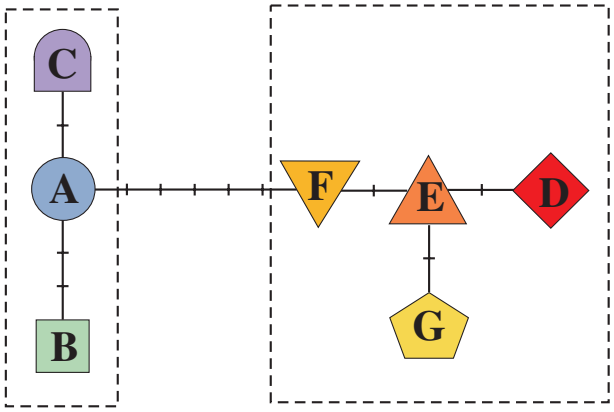

Figure 1 Minimum spanning tree of cpDNA haplotypes in *Pulsatilla vernalis*, based on the PCR–RFLP analysis, with the two major groups of haplotypes indicated by dashed lines.

whereas in the lowlands it occupies open habitats in pine forests and dry heathlands.

Pulsatilla vernalis is diploid ($2n = 16$), as reported from various parts of the distribution range (Aichele & Schwegler, 1957; Baumberger, 1971; Löve, 1980; Lauber & Wagner, 2000; Uotila, 2001). It has overwintering leaves, which distinguish this species from other European representatives of the genus. Aichele & Schwegler (1957) proposed four infraspecific varieties: var. *vernalis*, var. *bidgostiana*, var. *alpina* and var. *pyrenaica*, the two former ones occurring in lowlands and the two latter ones in alpine regions. Based on comparative morphology, some authors consider the eastern Asian *P. ajanensis* Regel & Tiling the closest relative of *P. vernalis* (Zāmels & Paegle, 1927; Scharfetter, 1953). Others, however, advocate a closer affinity of *P. vernalis* to species from section *Pulsatilla* (Aichele & Schwegler, 1957).

Self-compatibility has been observed at least in parts of the species' distribution area (Uotila, 2001), but flowers of *P. vernalis* are partially dichogamous (protogyny), which probably favours cross-pollination (Jonsson *et al.*, 1991). Hybrids of *P. vernalis* with other species of the section *Pulsatilla* have been observed (e.g. Aichele & Schwegler, 1957; Damboldt & Zimmermann, 1974; Uotila, 1980), but hybridization is usually restricted by partial geographical, altitudinal, ecological and phenological barriers. Decreased fertility of F1 hybrids also suggests the presence of post-zygotic internal barriers (Uotila, 1980).

Sampling

Sixty-one populations of *P. vernalis* spanning the whole species' range were sampled in 2001–03 (Table 1). Areas where more complex genetic patterns were expected (e.g. the Alps) were more densely covered. Five individuals per population were sampled. Plant material from each individual was dried in silica gel, and voucher specimens were collected for all populations from areas where *P. vernalis* is neither rare nor endangered. Vouchers were deposited in the herbarium of the Institute of Botany of the Polish Academy of Sciences in Kraków (KRAM).

In addition, 11 samples representing other European species of the genus *Pulsatilla* were sampled in the field or from herbarium material (Table 1) to estimate the level of interspecific differentiation of cpDNA and the potential influence of hybridization on the diversity of *P. vernalis* (to avoid confounding rare haplotypes with those resulting from interspecific gene flow). These outgroup species included all major European representatives of the genus belonging to the section *Pulsatilla* [*P. halleri* (All.) Willd., *P. montana* (Hoppe) Rchb., *P. patens* (L.) Mill., *P. pratensis* (L.) Mill., *P. slavica* G.Reuss, *P. vulgaris* Mill.], as well as a representative of *P. alpina* s.l. from the distant section *Preonanthus* [*P. alba* Rchb. = *P. alpina* (L.) Schrk. ssp. *alpicola* Rouy et Fouc.].

DNA isolation

Approximately 10 mg of dried plant tissue per sample was mechanically ground into fine powder, either manually after deep-freezing in liquid nitrogen or automatically using Mixer Mill 200 (Retsch, Haan, Germany) and 3-mm tungsten beads. Genomic DNA was then extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with one additional washing step. Some of the samples were additionally purified using either 7.5 M NH_4-Acetate or the Wizard DNA Clean-Up System (Promega, Madison, WI, USA). DNA extractions used for all samples had a concentration of *c.* 10–15 ng μL^{-1} (estimated on agarose gels against a dilution range of λ-DNA concentration standard).

Polymerase chain reaction–restriction fragment-length polymorphisms

Four samples were used in a pilot study for testing universal primers of 14 cpDNA regions (Taberlet *et al.*, 1991; Demesure *et al.*, 1995; Dumolin-Lapegue *et al.*, 1997; Shaw *et al.*, 2005). Annealing temperatures were tested in a gradient of 2°C steps using a PTC-200 gradient thermal cycler (MJ Research, Waltham, MA, USA). Some of the amplifications were also performed on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Seven cpDNA regions were successfully amplified and used for subsequent screening of polymorphism: *trn*H/*trn*K, *trn*K/*trn*K, *trn*C/*trn*D, *psb*C/*trn*S, *psa*A/*trn*S (Demesure *et al.*, 1995), *trn*L/*trn*F (Taberlet *et al.*, 1991) and *rpo*B/*trn*C (Shaw *et al.*, 2005). For four regions, published polymerase chain reaction (PCR) conditions were used, whereas for three longer fragments a slightly different protocol was followed (Table 2). This included an altered PCR mix with 0.5 μL of DNA and a reaction mix reaching the following final conditions in a total volume of 12.5 μL: 2 mM $MgCl_2$; 0.2 mM each dNTP; 0.2 μM each primer; 1.5 U of *Taq* Polymerase (Qiagen); 1 ng of bovine serum albumine (BSA). Cycling conditions were also modified as follows: 1 min at 94°C, followed by 10 cycles of 10 s at 94°C, 1 min at T_A (Table 2), 1 min per 1000-bp length at 68°C, followed by 25 cycles of 10 s at 94°C, 1 min at T_A, 1 min per 1000-bp length increasing by 10 s per cycle at 68°C, and a final elongation of 10 min at 68°C.

All successfully amplified regions were tested for restriction polymorphisms in single individuals chosen from 13 populations spanning major parts of the species distribution range, using 10 restriction enzymes (*Rsa*I, *Alu*I, *Hae*III, *Hap*II, *Hha*I, *Mse*I, *Hin*fI, *Bam*HI, *Eco*RI, *Hind*III: Amersham Biosciences, Piscataway, NJ, USA; New England Biolabs, Ipswich, UK). Reactions were carried out according to the manufacturers' suggestions in a total volume of 17 μL. Products were separated on 8% polyacrylamide gels in a cooled vertical electrophoresis system (Hoefer SE 600, Amersham Biosciences), and stained with ethidium bromide.

DNA Sequencing

Two cpDNA regions that were also included in the PCR–restriction fragment-length polymorphism (RFLP) analyses,

Table 1 Numbering, locality and cpDNA haplotypes of sampled populations of *Pulsatilla vernalis*. For cpDNA haplotypes, numbers in parentheses indicate the number of adenines in the poly-A stretch of the *trn*H-*psb*A sequence. The origins of outgroup plants are given at the bottom of the table.

No.	Country	Locality, collector	Altitude	Latitude/Longitude	Haplotype
1	A	Eastern Alps, Wölzer Alpen, Rettlkirchspitze (2475 m); PS	2415	47°15′ N/14°08′ E	A (10)
2	A	Eastern Alps, Hohe Tauern, Nussingkogel (2991 m); MR, PS	1995	47°02′ N/12°33′ E	A (12)
3	A	Eastern Alps, Gurktaler Alpen, Schoberriegel (2150 m); MR, PS, AT	2115	46°55′ N/13°53′ E	A (10)
4	A	Eastern Alps, Defereggen Gebirge, Stallersattel (2052 m); MR	2150	46°54′ N/12°12′ E	A (10, 12)
5	A	Eastern Alps, Ötztaler Alpen, Obergurgl, MR	2016	46°52′ N/11°02′ E	A (10, 12)
6	AND	Eastern Pyrenees, Port d'Envalira (2430 m); AR, MR	2469	42°33′ N/01°43′ E	A, E (10)
7	BG	Rila Mts., Goliam Blizniak (2779 m); MR, PK	2765	42°10′ N/23°35′ E	A (10)
8	CH	Western Alps, Alpes Vaudoises, Grand Chavalard (2899 m); MR	2056	46°10′ N/07°06′ E	A, D (10)
9	CH	Western Alps, Walliser Alpen, Visp, Oberi Hellela; AR, MR, PK	1600	46°16′ N/07°50′ E	A (10, 11)
10	CH	Central Alps, Rätische Alpen, Fluelapass (2388 m); AR, MR	1980	46°47′ N/09°55′ E	B (10)
11	CH	Central Alps, Urner Alpen, Furkapass (2431 m); AR, MR	2451	46°34′ N/08°25′ E	A (10, 13)
12	CH	Central Alps, Rätische Alpen, Julierpass (2284 m); MR	2210	46°28′ N/09°43′ E	B (10)
13	CH	Central Alps, Alpi Ticinesi, Nufenenpass (2478 m); AR, MR	2022	46°28′ N/08°25′ E	A (10, 12)
14	CZ	Sudetes, Krkonoše, Obři důl, Čertová zahrádka; JZ	1100	50°44′ N/15°43′ E	C (13)
15	D	Central Alps, Allgäuer Alpen, Jöchlspitze (2226 m); MS	1970	47°17′ N/10°22′ E	A (10)
16	D	Niederbayern, Siegenburg-Daßfeld; MS	-	48°43′ N/11°53′ E	A (10)
17	D	Oberpfalz, Köferinger Heide bei Amberg; MS	-	49°22′ N/11°55′ E	E (9)
18	D	Oberbayern, Pupplinger Au/Wolfratshausen; MS	-	47°52′ N/11°26′ E	A (10)
19	D	Niederbayern, Sandharlandener Heide; MS	-	48°47′ N/11°55′ E	A (10), E (9)
20	D	Oberpfalz, Trasgschieß bei Vohenstrauß; MS	-	49°37′ N/12°23′ E	E (9)
21	DK	Jylland, S of Holstebro,Vind Hede; AR, MR	90	56°16′ N/08°31′ E	E (9)
22	E	Western Pyrenees, Sierra de Chía, Casania (2372 m); AR, MR	1988	42°34′ N/00°25′ E	A (10)
23	E	Cordillera Cantàbrica, Peña Prieta (2536 m); AR, MR	2192	43°03′ N/04°44′ W	A (11)
24	F	Western Alps, Alpes Maritimes, Cime de Voga (2777 m); MR	2620	45°20′ N/06°50′ E	D (10)
25	F	Western Alps, Alpes de Haute-Provence, Col de Vars (2111 m); MR	2139	44°32′ N/06°42′ E	D (10)
26	F	Western Alps, Alpes Cottiennes, Col d'Izoard (2360 m); MR	2360	44°49′ N/06°44′ E	D (10)
27	F	Western Alps, Alpes Graies, Col d'Iseran (2764 m); MR	2425	45°24′ N/07°03′ E	A, D (10)
28	F	Western Alps, Alpes du Dauphiné, Col du Lautaret (2058 m); MR	2075	45°02′ N/06°24′ E	A, E (10)
29	F	Western Alps, Alpes du Dauphiné, Col du Gallibier (2646 m); MR	2478	45°04′ N/06°25′ E	A (10)
30	F	Eastern Pyrenees, Pic de Canigou (2784 m); PS, AT	2300	42°30′ N/02°26′ E	F (10)
31	F	Eastern Pyrenees, Puig Carlit (2921 m); AR, MR	2163	42°34′ N/01°59′ E	A (10, 11), E (10)
32	F	Central Pyrenees, Pic de Néouvielle (3091 m); AR, MR	2170	42°50′ N/00°08′ E	A (11), D (10)
33	F	Massif Central, Cévennes, Mont Lozère (1699 m); AR, MR	1526	44°26′ N/03°46′ E	A (11)
34	F	Massif Central, Mont Mézenc; AR, MR	1716	44°55′ N/04°11′ E	A (11)
35	F	Massif Central, Plomb du Cantal; AR, MR	1429	45°03′ N/02°48′ E	A (11)
36	FIN	South Savo, Anttola, Veeravuori; AR, MR	90	61°35′ N/27°40′ E	A (9, 12)
37	FIN	South Häme, Hausjärvi, Erkylä; AR, MR	130	60°43′ N/24°53′ E	A (12)
38	FIN	South Savo, Lappeenranta, Pontus, Pontuksenkaivanto; AR, MR	95	61°05′ N/28°18′ E	A (13)
39	FIN	South Savo, Luumäki, Pajari; AR, MR	110	60°54′ N/27°16′ E	A (12)
40	FIN	South Savo, Savonlinna, Inkerinkylä; AR, MR	95	61°51′ N/28°57′ E	A (12)
41	I	Central Alps, Dolomiti, Forcola di Coldose; PS, AT	2200	46°16′ N/11°38′ E	B (10)
42	I	Western Alps, Walliser Alpen, Gran San Bernardo (2469 m); MR	2170	45°52′ N/07°09′ E	A (10)
43	I	Western Alps, Alpes Graies, Piccolo San Bernardo (2188 m); MR	2116	45°42′ N/06°53′ E	D (10)
44	I	Western Alps, Alpi Cozie, Monte Viso (3841 m); MR, PS, AT	2672	44°39′ N/07°06′ E	A (12)
45	N	Jotunheimen, Galdhøpiggen (2469 m); AR, MR	1715	61°39′ N/08°23′ E	E (9)
46	N	Jotunheimen, Leirho (1178 m); AR, MR	1050	61°39′ N/08°09′ E	E (9)
47	N	Jotunheimen, Grønekinnkampen (1151 m); AR, MR	1090	61°17′ N/08°49′ E	E (9)
48	N	Rondane, Folldal; AS	-	62°08′ N/10°06′ E	E (9)
49	N	Dovrefjell, N of Hjerkinn; TB, TM	-	62°14′ N/09°33′ E	E (9)
50	N	Dovrefjell, S of Svåni; EF	-	62°17′ N/09°21′ E	E, G (9)
51	PL	Bory Tucholskie, vicinity of village Osie; AR, MR	95	53°38′ N/18°21′ E	D (10)
52	PL	Bory Tucholskie, vicinity of village Biała; AR, MR	108	53°41′ N/17°59′ E	D (10)
53	PL	Bory Tucholskie, vicinity of village Struga; AR, MR	110	53°46′ N/18°03′ E	E (9)
54	PL	Bory Tucholskie, vicinity of village Sokole-Kuźnica; AR, MR	89	53°25′ N/17°56′ E	D (10)

Table 1 Continued

No.	Country	Locality, collector	Altitude	Latitude/Longitude	Haplotype
55	PL	Bory Tucholskie, vicinity of village Ustroń; AR, MR	95	53°45′ N/18°01′ E	A (12)
56	PL	Łódź region, S from Bełchatów; AR, MR	190	51°17′ N/19°16′ E	A (10, 12)
57	PL	Góry Świętokrzyskie mts., Bocheniec near Chęciny; MR	240	50°48′ N/20°18′ E	A (9, 10)
58	PL	Western Carpathians, Tatry mts., Koszysta (2192 m); MR	2050	49°14′ N/20°03′ E	C (10)
59	PL	Western Carpathians, Tatry mts., Skrajna Turnia (2096 m); MR	1880	49°13′ N/20°00′ E	C (9)
60	PL	Western Carpathians, Tatry mts., Wrota Chałubińskiego; AR, MR	2025	49°12′ N/20°03′ E	C (9)
61	SK	Western Carpathians, Tatry mts., Vyšne Koprovské Sedlo; AR, MR	2080	49°10′ N/20°03′ E	E (9)
Outgroup samples					
O01	*P. alba* Rchb.	SK, Belanské Tatry; MR (2007, KRAM, *sine num.*)			
O02	*P. grandis* (Wender) Zam.	A, Niederösterreich, Weinviertel; WG (1983, HWG 17 895)			
O03	*P. halleri* (All.) Willd.	I, Aosta, Val Grauson; LS, WG (1983, HWG 18 779)			
O04	*P. montana* (Hoppe) Rchb.	I, Etsch-Tal, Bozen; WG (1959, HWG 4110)			
O05	*P. patens* (L.) Mill.	PL, Suwałki region, Sejny; MC (1964, KRAM 361 621)			
O06	*P. pratensis* (L.) Mill.	S, Öland; JC, WP (2006, KRAM, *sine num.*)			
O07	*P. pratensis* subsp. *nigricans* (Stoerck) Zam.	A, Niederösterreich, Wiener Wald; WG (1989, HWG 23 569)			
O08	*P. slavica* G. Reuss	SK, Trenčiansky kraj, Tematin; LM (1992, HWG *sine num.*)			
O09	*P. styriaca* (Pritz.) Simk.	A, Steiermark, Peggauer Wand, S Frohnleiten; WG (1960, HWG 6045)			
O10	*P. vulgaris* Mill.	Botanical Garden, Cracow, Poland; unknown provenience; MR (2006)			
O11	*P. vulgaris* Mill.	D, Bayern, SW Regensburg; WG (1990, HWG 25 406)			

Country abbreviations: A, Austria; AND, Andorra; BG, Bulgaria; CH, Switzerland; CZ, Czech Republic; D, Germany; DK, Denmark; E, Spain; F, France; FIN, Finland; I, Italy; N, Norway; PL, Poland; S, Sweden; SK, Slovakia. Collector abbreviations: AR, Anna Ronikier; AS, Anne-Cathrine Scheen; AT, Andreas Tribsch; EF, Eli Fremstad; JC, J. Cieślak; JZ, Jitka Zahradníková; LM, L. Mucina; LS, Louise Schratt; MC, M. Ciaciura; MR, Michał Ronikier; MS, Martin Scheuerer (DNA extracts made available by Harald Meimberg); PK, Philippe Küpfer; PS, Peter Schönswetter; TB, Tore Berg; TM, Thomas Marcussen; WG, Walter Gutermann; WP, W. Paul. HWG, herbarium W. Gutermann, Vienna.

Table 2 cpDNA fragments successfully amplified in *Pulsatilla vernalis*. Empirically selected annealing temperatures (T_A) are given for each fragment together with information on amplification details (Std, standard, published conditions: 1, Demesure *et al.*, 1995; 2, Taberlet *et al.*, 1991; 3, Shaw *et al.*, 2005; Long, altered conditions as described in the text; DMSO, concentration in the PCR mix), as well as approximate fragment lengths.

Name	T_A (°C)	PCR mix/program	DMSO	Length (bp)
*trn*H–*trn*K	60	Std; 1	–	1730
*trn*K–*trn*K	53.5	Long	2.5%	2700
*trn*C–*trn*D	58	Long	–	3500
*psb*C–*trn*S	57	Std; 1	–	1700
*psa*A–*trn*S	57	Long	2.5%	3700
*trn*L–*trn*F	55	Std; 2	–	1050
*rpo*B–*trn*C	53	Std; 3	–	1300

were further sequenced to test the reliability of the former data and to survey interspecific variation in *Pulsatilla*: the intergenic spacer between *trn*L(UAA) 3′-exon and *trn*F (Taberlet *et al.*, 1991), included in *trn*L–*trn*F region used for PCR–RFLP; and the *trn*H–*psb*A region (Hamilton, 1999), included in the *trn*H–*trn*K region used for PCR–RFLP. PuRe *Taq* Ready-To-Go PCR beads (Amersham Biosciences) were used for some of the amplifications, with 1 μL DNA and the following final conditions in a total volume of 25 μL: 1.5 mM $MgCl_2$; 0.2 mM each dNTP; 0.2 μM each primer; 2.5 U of puRe *Taq* DNA Polymerase; 1 μL of DMSO. The cycling program recommended by the manufacturer was used, with annealing temperatures of 50°C and 53°C for *trn*L–*trn*F and *trn*H–*psb*A, respectively. Alternatively, for some of the samples the amplification was performed with 0.5 μL of DNA extract and a reaction mix, reaching a total volume of 12.5 μL, and the following final conditions: 2 mM $MgCl_2$; 0.08 mM each dNTP; 0.2 μM each primer; 0.5 U of *Taq* Polymerase (Qiagen); 1 ng of BSA. In this case, the following cycling program was used: 4 min at 94°C, 35 cycles of 45 s at 92°C, 1 min at 50°C/53°C (*trn*L–*trn*F/*trn*H–*psb*A, respectively) and 45 s at 72°C, followed by a final extension of 10 min at 72°C. PCR products were purified using an EZNA Cycle-Pure Kit (Omega Bio-tek, Doraville, GA, USA) or a QIAquick PCR Purification Kit (Qiagen). Sequencing was performed using BigDye ver. 3.1 (Applied Biosystems) according to the manufacturer's protocol. Purified samples resuspended in 15 μL of formamide were separated on an ABI 3100-Avant capillary sequencer (Applied Biosystems) and analysed with the DNA Sequencing Analysis Software ver. 5.1 (Applied Biosystems). Both strands were sequenced to check for the reliability of detected differences.

In a pilot study, apart from plastid sequences, the ITS region from selected samples was amplified and sequenced using direct sequencing. However, the ITS sequences in *P. vernalis* displayed an additive pattern, namely double peaks in the electropherograms starting at some point of the sequence, suggesting the occurrence of multiple rDNA repeats of different length in the same genome. This behaviour, which has been recorded in other genera of the tribe (Miikeda *et al.*,

2006), prevented us from using ITS as a phylogeographical marker.

Data analysis

In the PCR–RFLP analysis, the lengths of uncut PCR products and restricted fragments were estimated using 100-bp and 1-kb DNA ladders (Fermentas, Burlington, Canada) in GENETOOLS ver. 3.02.00 (Syngene, Cambridge, UK). Restriction-site polymorphisms and length polymorphisms (indels) were treated as equally weighted single polymorphic sites, scored as present (1) or absent (0), and included in a binary matrix. A minimum spanning tree of haplotypes was constructed in ARLEQUIN ver. 2.0 (Schneider *et al.*, 2000).

DNA sequences were manually aligned using BIOEDIT ver. 5.0.9. (Hall, 1999). The relationships among haplotypes detected in *P. vernalis* and the outgroup taxa were analysed using the software TCS ver. 1.21 (Clement *et al.*, 2000). This program constructs haplotype networks, thus allowing loops and polytomies, by implementing the statistical parsimony algorithm described by Templeton *et al.* (1992). TCS was run with gaps coded as missing and indels recoded as binary characters so that they could be classed as a single event instead of as several independent events. An indel in *trn*L–*trn*F was coded into three instead of two character states. The poly-A stretch occurring in the *trn*H–*psb*A region was removed from the matrix as explained below.

RESULTS

Of the 70 DNA region/restriction enzyme combinations surveyed in the pilot study, 17 did not yield any digestion. Among the digestion patterns, only the *rpo*B–*trn*C region showed no polymorphism, with all other regions rendering polymorphisms. Fourteen fragment/enzyme combinations with clear and polymorphic digestion profiles were chosen for the analysis of the entire data set: *trn*H–*trn*K/*Hae*III, *trn*H–*trn*K/*Hap*II, *trn*H–*trn*K/*Mse*I, *trn*K–*trn*K/*Afa*I, *trn*K–*trn*K/*Bam*HI, *trn*K–*trn*K/*Mse*I, *trn*C–*trn*D/*Hae*III, *psb*C–*trn*S/*Alu*I, *psb*C–*trn*S/*Hae*III, *psb*C–*trn*S/*Mse*I, *psa*A–*trn*S/*Hha*I, *trn*L–*trn*F/*Eco*RI, *trn*L–*trn*F/*Hap*II, *trn*L–*trn*F/*Hha*I. Eleven polymorphisms were detected altogether across the whole data set, among which were six length polymorphisms (indels) and five restriction-site polymorphisms. They allowed the recognition of seven haplotypes within *P. vernalis* (A to G; Table 3).

Two main groups were distinguished among these seven haplotypes (Fig. 1); no alternative links between haplotypes enabling the construction of a minimum spanning network were revealed. The two haplotype groups were separated by five out of the 11 mutational steps detected. One group was composed of the most common haplotype, namely A, which also had the largest geographical distribution (frequency 0.53 in the total data set), and its local derivatives B and C. Haplotype B was restricted to three populations in the central Alps (Dolomites and Rhaetic Alps). Haplotype C was

Table 3 PCR–RFLP analysis of the cpDNA in *Pulsatilla vernalis*. Polymorphisms are given in relation to the most common haplotype (haplotype A). Length polymorphisms (indels) are presented as length differences. In the case of restriction-site polymorphisms, the respective enzyme is given along with its recognition sequence, followed by the character state. Polymorphism is presented as the number of restriction sites.

cpDNA region	Character state in haplotype A	Polymorphism	Haplotype						
			A	B	C	D	E	F	G
*trn*H–*trn*K	Length: 1730 bp	+10 bp	0	0	0	1	1	1	1
		+49 bp	0	0	0	0	0	0	1
	*Hae*III: 1 res [GG↓CC]	+1 res*	0	0	0	1	1	1	1
	*Hap*II: no cut [CC↓GG]								
*trn*K–*trn*K	Length: 2700 bp	+25 bp	0	0	0	1	1	0	1
	*Mse*I: 9 res [TT↓AA]	+1 res	0	0	0	1	1	1	1
*trn*C–*trn*D	Length: 3500 bp	+10 bp	0	0	1	0	0	0	0
*psb*C–*trn*S	*Mse*I: 5 res [TT↓AA]	+1 res	0	0	0	1	1	1	1
*psa*A–*trn*S	Length: 3700 bp	+20 bp	0	1	0	0	0	0	0
*trn*L–*trn*F	Length: 1050 bp	+50 bp	0	0	0	1	1	1	1
	*Hap*II: no cut [CC↓GG]	+1 res	0	0	0	1	0	0	0
	*Eco*RI: 2 res [G↓AATTC]	−1 res	0	1	0	0	0	0	0
Polymorphism			0	2	1	7	6	5	7

*The restriction polymorphisms generated by the two enzymes, *Hae*III and *Hap*II, are the result of one point mutation (as revealed by sequence analysis), treated as a single polymorphism.

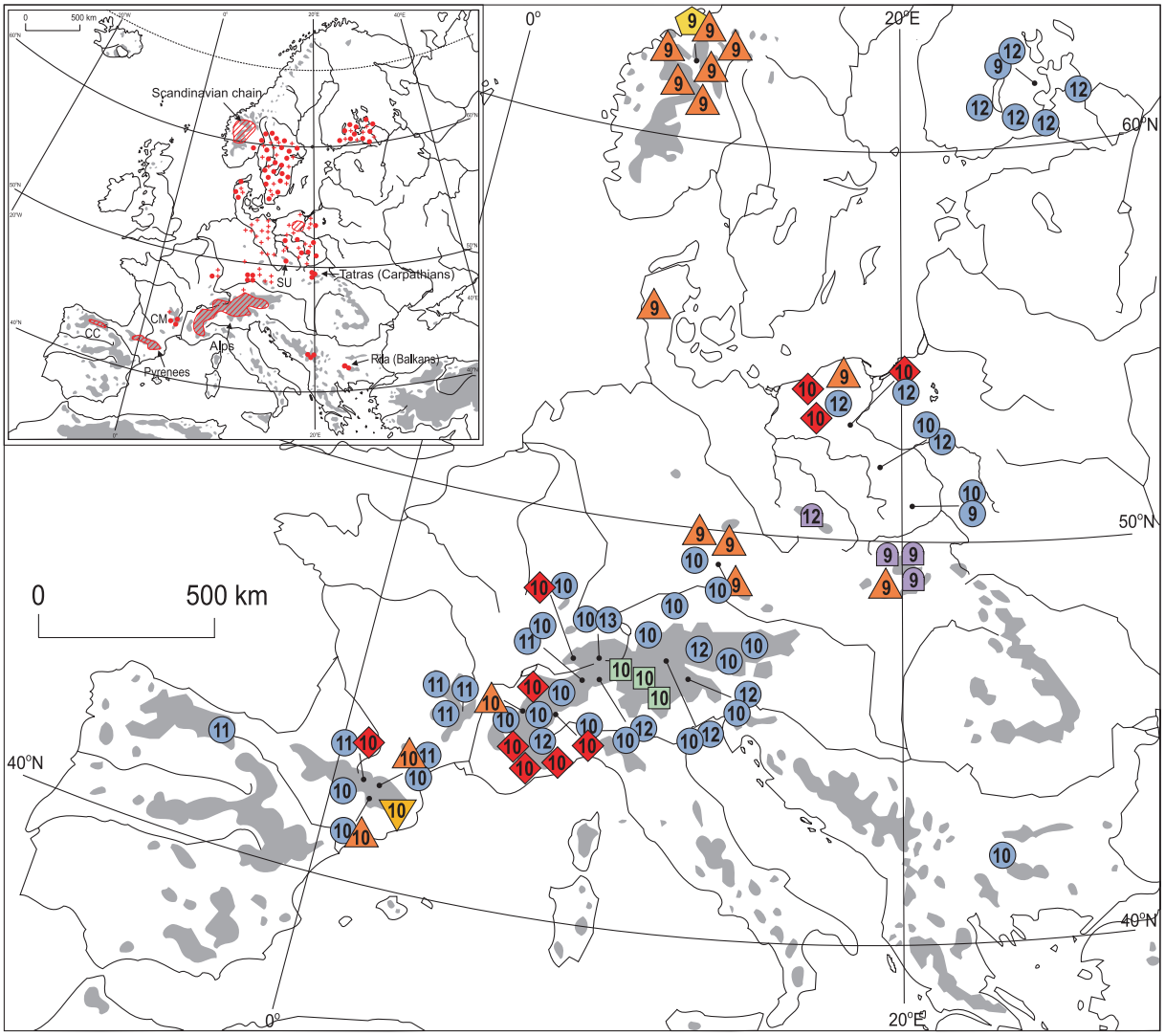

Figure 2 Geographical distribution of cpDNA haplotypes of *Pulsatilla vernalis* revealed by the PCR–RFLPs and sequencing. The colour and shape of haplotype designations on the map correspond to those given in Fig. 1. Numbers within the symbols give the character state of the poly-A stretch in the *trn*H–*psb*A sequence. In polymorphic populations, two overlapping symbols are used. Dark grey shading outlines European mountain areas. The insert presents a schematic distribution map of *P. vernalis*. Main mountain ranges are named (CC, Cordillera Cantábrica; CM, Central Massif; SU, Sudetes). Areas where the species is rare and declining are marked with dots and crosses.

identified in the Tatra mountains (western Carpathians) and the Sudetes. The second group was represented by haplotypes D and E (frequencies of 0.13 and 0.20, respectively), and the rare haplotypes F and G, found in single populations – F in one population from the Pyrenees and G in two plants from western Scandinavia (Fig. 2). The three most frequent haplotypes (A, D, E) are widely distributed across Europe. The second group of haplotypes (D–G), however, occurs mainly in the south-western Alps and the eastern Pyrenees, with a, presumably secondary, extension to the north. Populations from western and eastern Scandinavia were characterized by the fixed occurrence of haplotypes from different lineages: haplotype A in the east (Finland) and haplotype E in the west (Norway, Denmark). An additional haplotype G was found in two western Scandinavian plants, but its restricted distribution points to a post-glacial *in situ* length mutation.

Most populations of *P. vernalis* (75%) were fixed for single cpDNA haplotypes. Only nine populations harboured two different haplotypes (Fig. 2). These polymorphic populations occurred in potential contact zones between the two main haplotype groups, for example at the border between the south-western Alps and the rest of the Alps, in the eastern Pyrenees, and in the Polish lowlands.

Sequences of the *trn*L–*trn*F region were 501 bp long in all samples of *P. vernalis*. They contained two single nucleotide polymorphisms (point mutations), both between cytosine and guanine (Table 4). One point mutation was located in the recognition site of the *Hap*II enzyme (CC↓GG), at position

Table 4 Alignment of polymorphic fragments of the *trn*L–*trn*F sequence in *Pulsatilla vernalis* (haplotypes detected in the sequencing analysis are given; cf. Fig. 3) and other species of *Pulsatilla*. Two intraspecific nucleotide substitutions in *P. vernalis* are marked in boldface. The substitution of C by A at position 148 suppresses restriction by *Hap*II; the substitution of C by A at position 261 suppresses restriction by *Eco*RI (Table 3).

	Position of polymorphism in the alignment (bp)										
Species	043	065	090	091	106	148	207	261	285	306	437
P. vernalis (A)	C	TTAGTT	T	C	TT	**A**	———	**C**	———	ACAAACAAAAACTT	G
P. vernalis (B)	C	TTAGTT	T	C	TT	**A**	———	**A**	———	ACAAACAAAAACTT	G
P. vernalis (D)	C	TTAGTT	T	C	TT	**C**	———	**C**	———	ACAAACAAAAACTT	G
P. vernalis (EG)	C	TTAGTT	T	C	TT	**A**	———	**C**	———	ACAAACAAAAACTT	G
P. alba	C	———	G	A	–	**A**	TATATA	**C**	———	———	A
P. grandis	C	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G
P. halleri	A	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G
P. montana	C	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G
P. patens	C	TTAGTT	T	C	TT	**A**	———	**C**	TTAAT	ACAAACAAAAACTT	G
P. pratensis	C	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G
P. p. nigricans	C	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G
P. slavica	C	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G
P. styriaca	C	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G
P. vulgaris	C	TTAGTT	T	C	T-	**A**	———	**C**	———	ACAAAC———	G
P. vulgaris	C	TTAGTT	T	C	TT	**A**	———	**C**	———	———	G

148–151 of the sequence. The second point mutation was located in the recognition sequence of the *Eco* RI enzyme (G↓AATTC), at position 256–261 of the sequence. Both mutations were reliably detected in PCR–RFLPs (Table 3).

Most sequences of the *trn*H–*psb*A region had a length of 331–335 bp, but two individuals from a Scandinavian population had a 49-bp insertion, and therefore their sequences were 380 bp long (Table 5). In total, three polymorphic sites were found across *P. vernalis* in this region, including the above-mentioned insertion. The second polymorphism involved a single nucleotide mutation between guanine and thymine (at position 271 of the sequence). It was located in overlapping recognition sites of two enzymes, *Hap*II (CC↓GG) and *Hae*III (GG↓CC), and was also reliably detected in the PCR–RFLP analysis (Table 3). The third polymorphic site was a poly-A stretch with five distinct states found across populations (9–13 adenines). This variable poly-A repeat was not included in the overall cpDNA haplotype analysis, because of potential independent origins of the same state (Kelchner, 2000). It was separately analysed to enable us to search for additional geographical patterns at local scales (Fig. 2). In this analysis, each state (number of As) was treated as a separate character, as evolution of cpDNA microsatellites does not necessarily occur gradually in a stepwise way (e.g. Jakobsson *et al.*, 2007). Consistent patterns of the poly-A repeat provided valuable additional information in the case of haplotype E, characterized by a 10-A repeat in the Western European mountains and by a 9-A repeat in the lowland and Scandinavian populations. Predominance of an 11-A repeat also indicated a possible relationship between haplotype A from the Pyrenees and the Central Massif. Several states of the poly-A stretch were found in haplotype A in the Alps. 10-A and 12-A were the most frequent, although no clear spatial pattern was observed in plants bearing haplotype A (Fig. 2). One population had a unique 13-A repetition (Furka pass, Switzerland). Other alpine haplotypes (B, D) consistently had a 10-A repetition.

Considering the two cpDNA regions sequenced, *P. vernalis* did not share any haplotypes with other species (Fig. 3). The sequence of *P. alpina* s.l. from the section *Preonanthus* was distant from those for all other species and had seven specific characters (four nucleotide substitutions and three indels). Low cpDNA variation was observed throughout section *Pulsatilla*. Here, one large indel, one single nucleotide deletion, and one nucleotide substitution (in *P. halleri*) were found in the *trn*L–*trn*F sequence (Table 4). For the nucleotide polymorphism differentiating haplotypes in *P. vernalis* (A/C at position 148), the character state found in the most common haplotype A was also present in the remaining representatives of the section. One additional nucleotide substitution was found in the *trn*H–*psb*A sequence of *P. vulgaris*, and a single nucleotide insertion was found in some of the outgroup species (Table 5). For the nucleotide polymorphism (G/T at position 271) differentiating two main haplotype groups in *P. vernalis*, the character state found in haplotypes D, E and G was also found in the other species. In the haplotype network including all sequences, no external haplotypes grouped with those of *P. vernalis*. However, some of the outgroup taxa shared cpDNA haplotypes. Most network connections were unambiguous except for a loop provoked by an indel in *trn*L–*trn*F. The full 14-bp insertion was exclusive to *P. vernalis*, whereas the full deletion was present in the remaining samples except for one of *P. vulgaris*, which showed a partial indel in which the 5 bp on the 5′ end coincided with the insertion in

Table 5 Alignment of polymorphic fragments of the *trn*H–*psb*A sequence in *Pulsatilla vernalis* (haplotypes detected in the sequencing analysis are given, with poly-A stretch variants) and other species of *Pulsatilla*. The nucleotide substitution and 49-bp indel differentiating the haplotypes in *P. vernalis* are marked in boldface. Substitution of G by T at position 271 suppresses restriction by *Hap* II and *Hae* III (Table 3). The poly-A stretch excluded from the main analysis of haplotypes is marked in italics (the number of adenines per population is given in Table 1).

	Position of polymorphism in the alignment (bp)					
Species	012	086	140	195	201	271
P. vernalis (A;9A)	C	————————————————————————————————	T	-	*AAAAAAAAA*——	**T**
P. vernalis (AB;10A)	C	————————————————————————————————	T	-	*AAAAAAAAAA*—	**T**
P. vernalis (A;11A)	C	————————————————————————————————	T	-	*AAAAAAAAAAA*–	**T**
P. vernalis (A;12A)	C	————————————————————————————————	T	-	*AAAAAAAAAAAA*-	**T**
P. vernalis (A;13A)	C	————————————————————————————————	T	-	*AAAAAAAAAAAAA*	**T**
P. vernalis (E;9A)	C	————————————————————————————————	T	-	*AAAAAAAAA*——	**G**
P. vernalis (DE;10A)	C	————————————————————————————————	T	-	*AAAAAAAAAA*—	**G**
P. vernalis (G)	C	**TTTTTAGGAACATATTATCATATTATATTGGATTGGTTTATATATGTAC**	T	-	*AAAAAAAAA*——	**G**
P. alba	C	————————————————————————————————	C	T	*AAAAAAAAAA*—	**G**
P. grandis	C	————————————————————————————————	T	-	*AAAAAAAAAA*—	**G**
P. halleri	C	————————————————————————————————	T	T	*AAAAAAAAA*——	**G**
P. montana	C	————————————————————————————————	T	T	*AAAAAAAAAAAA*-	**G**
P. patens	C	————————————————————————————————	T	-	*AAAAAAAAA*——	**G**
P. pratensis	C	————————————————————————————————	T	-	*AAAAAAAAAAA*–	**G**
P. p. nigricans	C	————————————————————————————————	T	T	*AAAAAAAAAAAAA*	**G**
P. slavica	C	————————————————————————————————	T	-	*AAAAAAAAAA*—	**G**
P. styriaca	C	————————————————————————————————	T	-	*AAAAAAAAAA*—	**G**
P. vulgaris	T	————————————————————————————————	T	-	*AAAAAAAAAAAA*-	**G**
P. vulgaris	C	————————————————————————————————	T	-	*AAAAAAAAAA*—	**G**

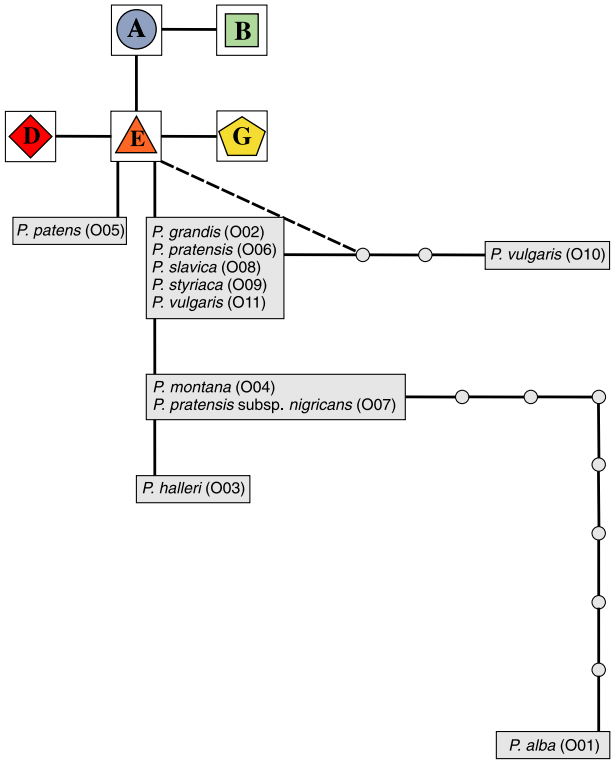

Figure 3 Haplotype network of cpDNA sequences of *Pulsatilla vernalis* and outgroup taxa (numbering of outgroup samples refers to those in Table 1). Haplotypes C and F of *P. vernalis* were detected only in the extended PCR–RFLP analysis and they are not represented in this network. Grey dots represent intermediate haplotypes missing in the data set.

P. vernalis (Table 4). All sequences are available from GenBank with accession numbers EF597126–EF597160 and EU285567–EU285570.

DISCUSSION

Main groups of haplotypes

Two distinct groups of haplotypes were distinguished in the analysis of cpDNA variation (Fig. 1). Both groups have a wide geographical distribution and neither shows a simple spatial pattern (Fig. 2). Given the low mutation rates in cpDNA (Wolfe *et al.*, 1987; Provan *et al.*, 1999), the genetic distance between these two main haplotype groups may well be the result of a relatively old differentiation predating the last glacial stage of the Quaternary glaciations. In such a scenario, phylogeographical traces left by such a differentiation would have been erased by subsequent glacial isolations and migrations. This conclusion fits the view of Merxmüller (1952) on the biogeography of European lineages: differentiation among alpine plant lineages has mainly ancient roots, whereas the last glaciation was important mostly in shaping their contemporary distribution. Isolation in refugia during the last glaciation is probably responsible for the observed spatial pattern by contributing some variation (for example spatially restricted haplotypes in some mountain ranges). Interestingly, the distribution of the haplotype groups does not coincide with the taxonomic varieties distinguished in *P. vernalis* – both groups were present in the ranges of all infraspecific taxa, suggesting their rather recent divergence or environmental influence through phenotypic plasticity.

In the comparison of cpDNA sequences across representatives of section *Pulsatilla*, low genetic divergence was observed among all taxa of section *Pulsatilla*, which is compatible with an ancient origin of the existing intraspecific variation, as it suggests low mutation rates not only within *P. vernalis* but also at the genus level. Only the sequence from the section *Preonanthus*, which has a distant position in the genus, exhibited a marked divergence. The generic-level data with the available outgroup sampling does not reveal historical hybridization events or incomplete ancestral lineage sorting, as *P. vernalis* did not share haplotypes with other taxa (Fig. 3). However, several outgroup species share the same haplotypes. A geographically extended sampling and larger comparative analysis of all taxa would be necessary to reconstruct the phylogenetic relationships between the species of *Pulsatilla*. This is beyond the scope of this study.

Although no clear overall geographical pattern was found for the two main cpDNA haplotype lineages, presumably because of their ancient origin, regional patterns of haplotypes could be detected. These regional patterns affecting the European mountain system and corresponding lowlands are discussed below.

The Alps

Chloroplast DNA variation in the Alpine populations of *P. vernalis* reveals a clear differentiation between the southwestern Alps and the central/eastern Alps (Fig. 2). Populations in the former were dominated by haplotype D, whereas the latter were characterized by haplotype A or its close derivative B. The eastern distribution border of haplotype D was on the Aosta valley and the western Swiss Valais region, where the sampled population was polymorphic with respect to cpDNA haplotypes (D and A). Haplotypes from the two areas were separated by seven mutational steps (Fig. 1). Haplotype B was geographically restricted to three populations in the Dolomites and in the Rhaetic Alps in eastern Switzerland.

A considerable effort has been invested in studying the genetic diversity of alpine plants and in elucidating the history of the Alpine flora during and after the Quaternary glaciations, in particular regarding aspects such as glacial survival and recolonization (Gugerli & Holderegger, 2001). Most species analysed with molecular markers revealed a strong division into western and eastern Alpine clusters, further differentiated into additional subgroups across the Alps, for example *Androsace alpina*, *Phyteuma globulariifolium* and *Ranunculus glacialis* (Schönswetter *et al.*, 2002, 2003, 2004a). A clear differentiation of a western Alpine group was also detected in *Comastoma tenellum* (Schönswetter *et al.*, 2004b), but with little diversification in the central and eastern Alps. In fact, the

phylogeographical pattern of this species in the Alps was very similar to that of *P. vernalis*, except for the occurrence in the latter of an additional spatially restricted haplotype (B) in the central Alps. The geographical border of the western Alpine cpDNA haplotype of *P. vernalis* (haplotype D) is highly congruent with that of the westernmost phylogeographical groups found in the aforementioned taxa. Taken together, available data indicate strong differentiation of populations from the south-western Alps, caused by an isolated peripheral refugium in this region. Interestingly, this area is also the richest in the Alps in terms of endemics, characterized by, among others, several local palaeoendemic species (Pawłowski, 1970). All this testifies to a specific history for the south-western Alps.

However, a lack of phylogeographical structure in the Alps was also found, for example in *Oxytropis campestris* (Schönswetter *et al.*, 2004c) and *Saxifraga oppositifolia* (Holderegger *et al.*, 2002). *Pulsatilla vernalis* and *Comastoma tenellum* also show a weak structure in the central and eastern Alps. Interestingly, all these species represent alpine plants that also have a tendency to grow at subalpine or even lower altitudes (Aeschimann *et al.*, 2004), and thus with a wider ecological niche than obligate alpine species, such as *Androsace alpina*, *Phyteuma globulariifolium* and *Ranunculus glacialis*. The highly structured genetic variation found across the Alps in the latter three high alpine species (reviewed by Schönswetter *et al.*, 2005) may be a result of differentiation in small isolated alpine refugia at the southern edge of the Alps, whereas facultative alpine plants with wider ecological requirements survived in peripheral less isolated refugia with higher possibilities of genetic exchange, which may have resulted in weaker differentiation across the Alps. However, survival in isolated refugia during the last glaciation may be responsible for some genetic structuring in *P. vernalis* in the central Alps. Haplotype B could be linked with glacial survival in a refugium in the Dolomites, which supports the results of other studies suggesting the existence of a glacial refugium in this area (Schönswetter *et al.*, 2005).

Other parts of the European alpine system

The cpDNA data from *P. vernalis* showed a relationship between the eastern Pyrenees and the south-western Alps, reflected by the presence of haplotype D in the south-western Alpine populations and of haplotypes D and the closely related E and F in the eastern part of the Pyreneo-Cantabrian range of the species (Fig. 2). A close relationship between the Pyrenean and western Alpine populations was also found in *Phyteuma globulariifolium*, for which AFLP data suggest a relatively recent immigration to the eastern Pyrenees from the western Alps (Schönswetter *et al.*, 2002). In *P. vernalis*, however, haplotypes of the westernmost populations, including those from the Cordillera Cantábrica and central Pyrenees, harboured haplotype A (with a specific 11-A repeat in the *trn*H–*psb*A sequence), representing the other genetic group. Similar complex patterns of diversity between the Alps and the Pyrenees have been found in *Soldanella alpina* and *Saxifraga oppositifolia* (Zhang *et al.*, 2001; Vargas, 2003), possibly pointing to long-distance dispersal or several colonization events with a redistribution of ancient genotypes.

Populations of *P. vernalis* from the Central Massif were characterized by the most common haplotype, A, present in both the Alps and the Pyrenees. However, they harboured an 11-A repeat in the *trn*H–*psb*A sequence, which also occurred in the Pyrenean and Cantabrian populations with haplotype A (Fig. 2). In the Alps, the 11-A repeat was only found in one distant population of the central Swiss Alps (possibly of polytopic origin). Single-nucleotide repeats are to be treated cautiously in phylogeographical considerations, but a recent European-wide study of *Fraxinus excelsior*, based on chloroplast microsatellites, showed that phylogeographical inference based on these repeats was reliable despite potential homoplasy (Heuertz *et al.*, 2004). The above data on *P. vernalis* could, therefore, suggest a link between the Central Massif populations and those in the central Pyrenees and Cantabrian mountains. The Central Massif could have acted as a refugial area for *P. vernalis*, as has been proposed for *Calluna vulgaris*, which exhibited the highest cpDNA variation in the Cévennes (Rendell & Ennos, 2002).

A specific haplotype, C, was found at the eastern edge of the European alpine system, in the Tatras (western Carpathians) and the Sudetes, indicating that these populations did not arise from recent dispersal, but were isolated from the Alps. This study also suggests a closer relationship between populations from the Tatra and Sudety mountains than is usually admitted (cf. Pawłowski, 1977). The genetic distinctiveness of the high-mountain flora of the Tatras and the Alps has also been observed in other species, for example in *Ranunculus glacialis* (Schönswetter *et al.*, 2003) and *Campanula alpina* (Ronikier *et al.*, 2008). All these data point to a long isolation of the alpine flora in the Tatra mountains. A close relationship of the north-eastern Alpine populations with populations in the western Carpathians, however, was shown for *Pritzelago alpina* (Kropf *et al.*, 2003), possibly linked to a gradual colonization of the European mountain system from the Cantabrian and the Mediterranean area. Relatively recent colonization of the Alps from the east through the Tatra mountains was also suggested for the arctic-alpine *Ranunculus pygmaeus* (Schönswetter *et al.*, 2006).

Interestingly, one population of *P. vernalis* in the Tatra mountains contained haplotype E, which could be a result of recent dispersal from populations of a lowland lineage. Such long-distance dispersal events may occur relatively often (e.g. Brochmann *et al.*, 2003; Schönswetter *et al.*, 2004b) and have a substantial impact on shaping the genetic variation of plant populations (Trakhtenbrot *et al.*, 2005).

The south-eastern accession of *P. vernalis* from the Bulgarian Rila mountains (the Balkans) was characterized by the common haplotype A with a 10-A repetition in the *trn*H–*psb*A sequence, characteristic for most populations in the Alps. Hence, cpDNA does not support a strong differentiation of the Balkan populations of *P. vernalis*, despite their isolation.

Furthermore, this population was more closely related genetically to the Alps than to the Carpathians (Tatra mountains).

The lowland areas and Scandinavia – northern refugia?

Although *P. vernalis* is clearly an alpine species, with large populations above 2500 m a.s.l. in the Alps, it also has a marked tendency to occupy subalpine and dry steppe-like habitats (Favarger, 1995; Aeschimann *et al.*, 2004). Its contemporary distribution range points to the alpine belt as its primary habitat (Scharfetter, 1953). The higher diversity of haplotypes in mountain areas compared with that in lowland populations supports the opinion that *P. vernalis* originated in alpine habitats and secondarily colonized the lowland areas during glacial climate changes.

Both main genetic groups are present in the lowland part of the distribution range of *P. vernalis*, located to the north of the European alpine system. The distribution of haplotypes suggests that the colonization of lowlands by these two major lineages has occurred independently, with subsequent contact zones in the German and Polish lowlands. Lowland populations in Poland and Germany are probably the source of further northward migration after the retreat of the Weichselian ice sheet from the South of Fennoscandia.

The western part of the lowland range could have been colonized by a lineage migrating from the western Alps/eastern Pyrenees area. Interestingly, all lowland and Scandinavian populations bearing haplotype E have a specific 9-A repeat in the *trn*H–*psb*A sequence, whereas contemporary populations with this haplotype in the Alps and the Pyrenees have a 10-A repeat (Fig. 2). The distinctiveness of haplotype E in this part of the species' distribution and its predominance over a close haplotype D (characterizing the western Alps) suggest that the lowland area is not the result of a recent, post-glacial (re)colonization. It could indicate an earlier migration from mountain to lowland areas followed by isolated survival in northern periglacial refugia, located between the Alps in the south and the Scandinavian ice sheet in the north, in an area then covered by tundra and cold steppe vegetation (Mojski, 1993). Range expansion from a periglacial northern refugium would better explain the almost monomorphic pattern of the western Scandinavian lineage. The same could also be hypothesized for the second main lineage, although it does not present such a straightforward pattern. As *P. vernalis* is able to colonize both alpine grasslands and dry heathlands, it could have been present in the lowlands during glaciations. The presence, based on macrofossil data, of 'cryptic' northern refugia during the last glacial maximum has been suggested even for tree species (Stewart & Lister, 2001; Willis & van Andel, 2004). Fossil remains of plants found in different areas and their assemblages show that vegetation in unglaciated central Europe was more diverse than is often thought. Glacial survival in refugia at the edges of the Scandinavian ice sheet has also been proposed for *Calluna vulgaris* (Rendell & Ennos, 2002), based on a comparatively high diversity of cpDNA haplotypes in Scandinavian populations and the lack of a south-based colonization lineage.

This phylogeographical study of *P. vernalis* provides good support for the scenario of the independent (re)colonization history of eastern Scandinavia (Finland) and western Scandinavia (Sweden and Norway), possibly with marked founder effects reflected by the fixed character of haplotypes in both groups. However, alternative scenarios have been inferred from other plant species, such as a localized founder event from the eastern Alps to Scandinavia in *Ranunculus glacialis* (Schönswetter *et al.*, 2003), close links with the Carpathians in *Trollius europaeus* (Després *et al.*, 2002) and *Comastoma tenellum* (Schönswetter *et al.*, 2004b), or partially intermixed lineages in *Quercus* (Ferris *et al.*, 1998; Petit *et al.*, 2002) as well as in *Carex digitata* and *Melica nutans* (Tyler *et al.*, 2002), demonstrating the complexity of the migration patterns that have shaped the contemporary Scandinavian flora.

During the Holocene, *P. vernalis* survived in the lowlands in sparse pine forests and heathlands. Here, it has been considered as a preboreal (*c.* 10,000 yr BP) relict (Szafer, 1977; Muller, 1997), but – as suggested by our data – the presence of this species in the lowlands could be much older. In the lowland part of the distribution area, *P. vernalis* was historically more abundant than it is at present, and a strong decline has been observed during the last century. This decline of populations is correlated with anthropogenic disturbance of habitats and changes of land use, but these factors could act synergistically with the low disturbance tolerance of the species at the edge of its ecological niche.

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