SPECIATION

Effects of Pleistocene glaciations on the genetic structure of *Saxifraga florulenta* (Saxifragaceae), a rare endemic of the Maritime Alps

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The high species diversity and endemism of the Maritime Alps suggest that this region may have held several refugia during the Pleistocene glaciations. Nevertheless, this assumption has rarely been examined. Here we investigate the genetic diversity of *Saxifraga florulenta*, a rare endemic restricted to siliceous substrates in the Maritime Alps. Overlaying the maximum extension of the ice sheet during the Pleistocene, the current distributions of *S. florulenta* and siliceous substrates suggest the existence of two putative refugial areas in the Maritime Alps. By using evidence from amplified fragment length polymorphisms we aim at elucidating whether genetic structure of the species corresponds to this two-refugia hypothesis and how this genetic information can be used to ensure its long-term conservation. Low levels of species-wide and within-population genetic diversity were detected, suggesting strong historical bottlenecks. Bayesian and principal coordinate analyses identified two population groups in agreement with the two refugia hypothesis. However, weak genetic divergence between these groups suggests that their separation happened more recently, and that *S. florulenta* survived the Pleistocene glaciations in one main refugium. The lack of a significant correlation among genetic and geographic distances implies that populations are not at migration-drift equilibrium and current levels of gene flow among them do not appear to be sufficient to balance the effect of genetic drift. Hence, in future conservation strategies, special care should be taken to preserve both gene pools and prevent further fragmentation of populations.

KEYWORDS: conservation, endemism, genetic diversity, Maritime Alps, Pleistocene glaciations, *Saxifraga florulenta*

INTRODUCTION

The progressive climate cooling of the Neogene, culminating in the glacial episodes of the Pleistocene, profoundly altered the composition and distribution of the European alpine flora (Bennett & al., 1991; Lang, 1994; Ehlers & Gibbard, 2004). Glaciers repeatedly covered the Alps, promoting the extinction of many Tertiary taxa, while a few unglaciated areas (i.e., refugia) at the centre and periphery of the Alpine chain allowed the survival of others (Comes & Kadereit, 1998; Stehlik, 2003; Tribsch & Schönswetter, 2003; Schönswetter & al., 2005). The cyclic climatic shifts of the Pleistocene caused the repeated fragmentation and isolation of populations in glacial refugia, strongly influencing the genetic structure of surviving species (Hewitt, 1996, 2000; Comes & Kadereit, 1998, 2003; Taberlet & Cheddadi, 2002; Tribsch & Schönswetter, 2003).

Until now, most studies of infra-specific genetic variation in Alpine plants focused on the central (Holderegger & al., 2002; Stehlik & al., 2002a,b) and eastern Alps (Tribsch & al., 2002; Tribsch & Schönswetter, 2003; Schönswetter & al., 2004b, 2006a), where several refugial areas, including ice-free cliffs, valleys, and nunataks (i.e., ice-free mountain tops), have been identified (reviewed by Schönswetter & al., 2005). However, equivalent studies on the south-western Alps, and, more specifically, the Maritime Alps, remain surprisingly scarce (but see Gaudeul & al., 2000; Diadema & al., 2005; Minuto & al., 2006), despite the recognised importance of this region as a cradle of alpine biodiversity (Barbero, 1967; Médail & Quézel, 1997; Médail & Verlaque, 1997; Casazza & al., 2005). Further population genetic surveys on species endemic to the Maritime Alps are thus necessary to understand the evolutionary processes that produced the current high levels of endemism and overall species richness in this region.

The Maritime Alps, extending for about 190 km along the French-Italian border between Nice and Cuneo (Fig. 1A, B), have been recognised as the portion of the Alpine chain that comprises the highest number of species (2,900) and, specifically, endemics (68) per square kilometre (Casazza & al., 2005). Owing in part to the buffering influence of the Mediterranean Sea on local climate, several ice-free areas may have allowed the local survival of Tertiary elements during the Pleistocene glacial maxima (Ozenda, 1950; Pawlowski, 1970; Martini, 1982, 1992; Médail & Verlaque, 1997). Additionally, this segment

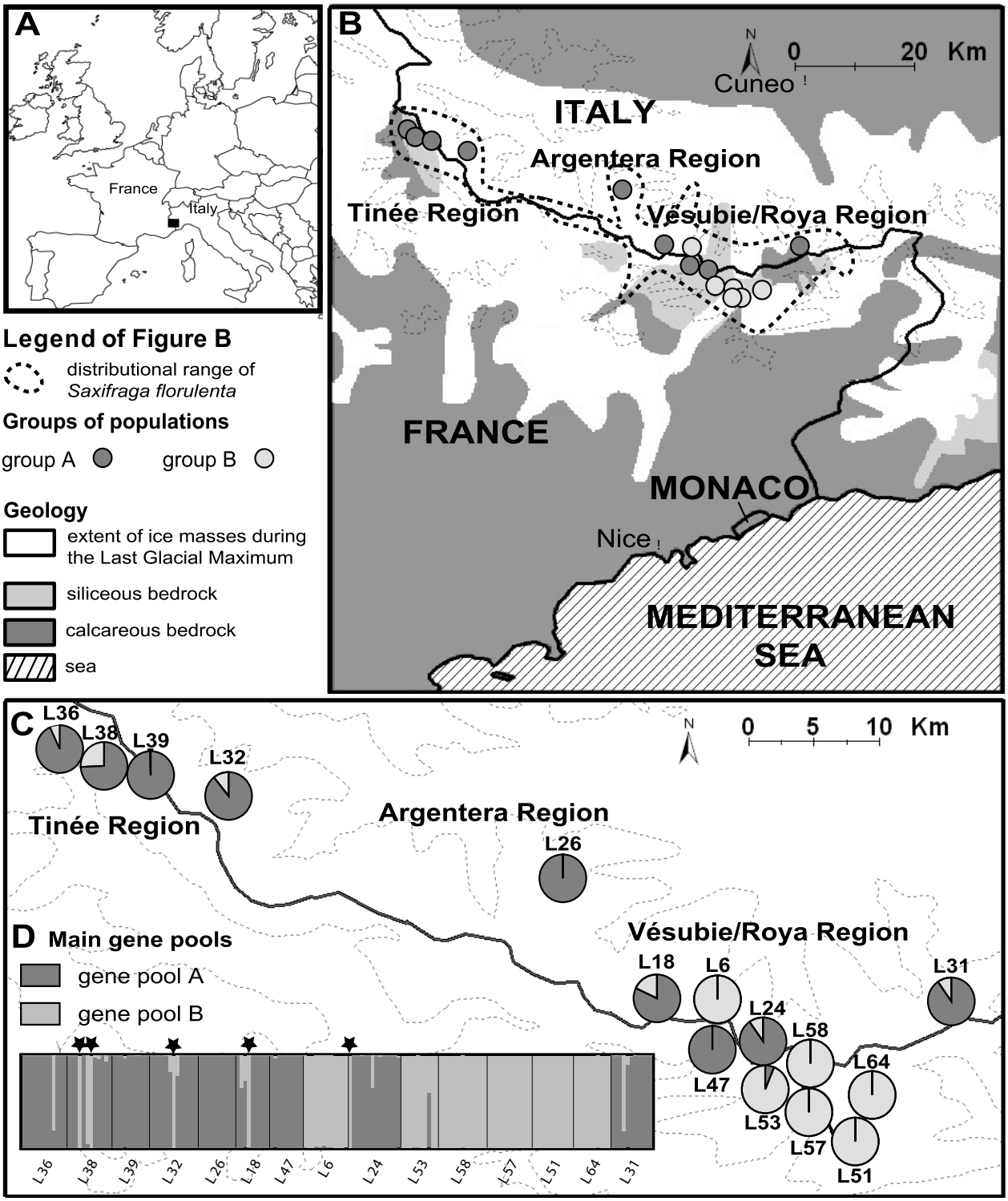

Fig. 1. A, The position of the Maritime Alps within Europe is indicated by a black square. B, Geographical location of the 15 populations sampled from the distributional range of *Saxifraga florulenta*. Populations were assigned to group A or B based on results from Bayesian clustering and Principal Coordinates analyses. The maximum extent of the ice masses during the Last Glacial Maximum and geological substrates are indicated (modified from Diadema & al., 2005). Dotted lines represent the 2,000 m limit. C, Proportion of the gene pools for each population (shown as pie diagrams) estimated by the Bayesian clustering analysis. Dotted lines represent the 2,000 m limit. D, Proportional membership of each individual to the inferred gene pools estimated by the Bayesian clustering analysis. Each individual is represented by a single vertical bar. First generation immigrants are marked by asterisks. Population codes are shown at the bottom of the graph.

of the Alps is characterised by a remarkable mixture of substrates—ranging from metamorphic to sedimentary rocks (Fig. 1B)—and a broad spectrum of vegetational zones—ranging from mediterranean to alpine—further contributing to its species richness (Médail & Verlaque, 1997; Casazza & al., 2008).

The focal species of the study presented here, *Saxifraga florulenta* Moretti (Saxifragaceae), is endemic to an area of about 100 km^2 centred around the granitic Mercantour massif of the Maritime Alps and occurs exclusively on siliceous, vertical cliffs at the highest altitudinal zone, above 2,000 m (Fig. 1B; Filipello & Gardini-Peccenini, 1985; Webb & Gornall, 1989). The relatively low number of populations (approximately 100; Focquet & Romain, 1988) and individuals per populations (ranging between less than 10 and 300; Arroyo pers. obs.) determined the official listing of *S. florulenta* as "rare" (Walter & Gillett, 1998).

The long-lived perennial, diploid *S. florulenta* (n = 14) is normally semelparous, a rare life-history strategy reported only rarely in other European saxifrages (Webb & Gornall, 1989; Holderegger, 1996). Detailed knowledge of the reproductive biology of *S. florulenta* is not available, except for general observations at the genus level suggesting that the radially symmetric, unspecialised flowers are visited by insects, and that the multi-flowered inflorescences might allow selfing via geitonogamy (Webb & Gornall, 1989). Flowers are usually tricarpellate, a feature that differs starkly from the bicarpellate condition typical in *Saxifraga* s.str. Morphological variation is virtually absent in the species, which is not known to form hybrids (Bland, 2000). The distinctive floral morphology and life history of *S. florulenta* prompted its separation in subsect. *Florulentae* (Engler & Irmscher) Gornall, within sect. *Ligulatae* (Webb & Gornall, 1989). Recent molecular phylogenetic analyses, however, suggested that it is more closely related to members of sect. *Porphyrion* (Conti & al., 1999). The isolated taxonomic placement and narrow distribution of *S. florulenta* induced several botanists to consider it a Tertiary relict that survived the Pleistocene glaciations in refugia of the Maritime Alps (Martini, 1982, 1992; Grey-Wilson, 1985).

The glacial history of the Maritime Alps is complex and still poorly understood at high levels of spatial resolution. However, available data allow the delimitation of two major geographic areas that putatively remained to be ice-free over the climatic oscillations of the Quaternary (Hughes & al., 2006). During the last glacial maximum, most of the Maritime Alps were covered by ice masses, except for two major siliceous nunataks, which might have enabled the persistence of the edaphically specialised *S. florulenta* (Fig. 1A; M. Dubar, CNRS, France, unpub. res.; see also Diadema & al., 2005). Indeed, when the maximum extension of the ice sheet during the Riss glaciation (ca. 130,000 yrs. ago) is overlaid with the occurrence of siliceous substrates and the current distribution of *S. florulenta*, a minimum of two main refugial areas can be proposed: the Tinée region and the Vésubie/Roya region (Fig. 1) (Filipello & Gardini-Peccenini, 1985; Webb & Gornall, 1989). The main goal of the present study is to use evidence from amplified fragment length polymorphism (AFLP) fingerprinting to elucidate the effects of Pleistocene climate changes and ongoing population processes on the genetic structure of *S. florulenta*. In particular we aimed at addressing the following questions: *(1)* Does the level and distribution of genetic variability in *S. florulenta* support the hypothesis of *in situ* survival in two refugia (i.e., the Tinée region and the Vésubie/Roya region) of the Maritime Alps? *(2)* How is genetic variation partitioned among and within populations? *(3)* How can this genetic information be used to ensure the long-term conservation of the species?

MATERIALS AND METHODS

Taxon sampling and DNA extraction. — A total of 168 plant accessions, representing 15 populations, were sampled to cover the distributional range of *S. florulenta* (Fig. 1B). For each population, leaf material from 9 to 14 individuals was collected, dried in silica gel and stored at 4°C (Table 1). Total DNA was extracted with the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

AFLP protocol. — The AFLP procedure followed Vos & al. (1995), with some modifications. DNA (35 ng) was digested in 50 µl volume with 1 Unit EcoRI and 1 Unit MseI (New England Biolabs, Beverly, Massachusetts, U.S.A.). Forty microliters of digested DNA were ligated overnight using 1 Unit T4 DNA ligase (Promega, Madison, Wisconsin, U.S.A.), 3 µM MseI- and 0.3 µM EcoRI-adapters (Microsynth, Balgach, Switzerland). The pre-selective amplification was performed using 2 µl of 10-fold diluted, ligated DNA, 2.5 mM $MgCl_2$, 0.5 Unit *Taq* DNA Polymerase (Promega), 1.5 µM of each pre-selective primer (EcoRI.A and MseI.C, Microsynth) and 0.375 mM dNTPs in 20 µl with the following PCR profile: 72°C/2 min, 94°C/2 min, 30 cycles of 94°C/45 s, 56°C/45 s and 72°C/2 min, with a final extension at 72°C/10 min. The selective PCR was performed using 5 µl of the 20-fold diluted pre-selective PCR in a 15 µl reaction containing 2.5 mM $MgCl_2$, 0.5 Unit of *Taq* DNA polymerase (Promega), 1.5 µM fluorescent EcoRI primer (EcoRI.AXX), 1.5 µM MseI primer (MseI.CXX; Microsynth), and 0.375 mM dNTPs with the following PCR profile: 95°C/10 min, 35 cycles of 94°C/30 s, 1 min annealing (starting at 65°C, reduced by 0.7°C for 13 cycles and maintained at 56°C for 22 cycles) and 1 min elongation at 72°C, with a final extension at 72°C/10 min. 1.5 µl of

the selective amplification along with the size standard (GeneScan™ 400HD [Rox™ Dye] Standard, Applied Biosystems, California, U.S.A.) were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). AFLP patterns were scored using the program Genographer (version 1.6.0, Montana State University 2001; http://hordeum.oscs.montana.edu/genographer). Only unambiguous markers were scored.

The influence of different DNA concentrations on the reproducibility and intensity of AFLP markers was tested by carrying out the entire AFLP protocol twice on six individuals from six different populations using four different concentrations of total DNA extracts (1.625, 3.125, 6.25 and 12.5 ng DNA/µl) for two primer combinations (EcoRI.AGT/MseI.CAA, EcoRI.AGT/MseI.CAG). Since all amplifications showed reproducibility higher than 95%, all extractions with a concentration higher than 1.625 ng DNA/µl were used (data not shown). Sixty-four selective primer pairs were tested for the clarity of produced peaks and presence of variability on seven individuals from different populations. Four selective primer pairs (Table 2) with the highest variability and reproducibility were chosen for final analysis.

Data analyses. — Bayesian analyses of population structure were performed on AFLP markers using STRUCTURE-v2.2.2 (Pritchard & al., 2000; Falush & al., 2003, 2007). Ten independent runs were carried out for values of K (the number of ancestral gene pools) ranging from 1 to 10 with 10^6 Markov chain iterations following an initial burn-in of 10^5 iterations and using the admixture model with allele frequencies correlated within populations (Falush & al., 2003). The most likely value for K was determined by calculating the change in the ln likelihood (ΔK) of the genetic data given the number of ancestral gene pools (Pr(X | K)) (Evanno & al., 2005). Populations were split into group A and group B populations based on their dominant ancestral gene pool component. To identify recent immigrants or individuals with immigrant ancestry in the last

Table 1. Population code, locality, estimated population size, number of individuals sampled and genetic characteristics of populations used in this study.

Pop. code	Locality	Est. pop. size	Sample size	$\%P_{pop}$	H_s (95%HPD)	$H_{e\ all}$ (SE)	$H_{e\ only\ polym.}$ (SE)	F (90%HPD)	M	Dominant gene pool
L36	Lacs de Morgon	80	12	20.69	0.138 (0.112–0.173)	0.076 (0.015)	0.140 (0.024)	0.267 (0.194–0.432)	0.686	A
L38	Maison Forestiere de Tortisse	50	12	25.00	0.155 (0.130–0.188)	0.087 (0.015)	0.161 (0.025)	0.186 (0.125–0.326)	1.092	A
L39	Refuge de Vens	65	10	25.00	0.163 (0.135–0.196)	0.091 (0.016)	0.168 (0.026)	0.218 (0.069–0.260)	0.894	A
L32	Rifugio Zanotti	24	13	28.45	0.181 (0.149–0.220)	0.115 (0.018)	0.211 (0.027)	0.137 (0.077–0.236)	1.574	A
L26	Gias Gros	60	10	21.55	0.144 (0.114–0.182)	0.085 (0.016)	0.157 (0.026)	0.207 (0.187–0.415)	0.955	A
L18	Rifugio Remondino	200	9	16.38	0.131 (0.107–0.160)	0.051 (0.012)	0.094 (0.021)	0.387 (0.160–0.425)	0.396	A
L47	Valette Escure	60	9	14.66	0.117 (0.091–0.151)	0.046 (0.012)	0.085 (0.021)	0.335 (0.296–0.562)	0.496	A
L24	Ricovero Lombarda	60	14	23.28	0.136 (0.105–0.170)	0.079 (0.015)	0.146 (0.024)	0.384 (0.217–0.437)	0.401	A
L31	Palanfré, Lago de Frisson	30	11	19.83	0.143 (0.121–0.170)	0.063 (0.013)	0.116 (0.023)	0.257 (0.200–0.423)	0.722	A
L6	Bacino artificiale di Chiotas	80	12	10.34	0.108 (0.079–0.146)	0.033 (0.010)	0.060 (0.018)	0.628 (0.463–0.735)	0.148	B
L53	Vallon Cabret	180	10	18.10	0.146 (0.118–0.182)	0.062 (0.014)	0.114 (0.023)	0.296 (0.269–0.516)	0.595	B
L58	Lac de la Fous	70	13	18.97	0.143 (0.109–0.188)	0.073 (0.015)	0.134 (0.025)	0.497 (0.371–0.614)	0.253	B
L57	Mur des Italiens	70	12	12.07	0.129 (0.092–0.177)	0.049 (0.013)	0.091 (0.022)	0.584 (0.506–0.746)	0.178	B
L51	Vallon de l'Autier	77	11	17.24	0.146 (0.109–0.190)	0.069 (0.015)	0.128 (0.026)	0.557 (0.368–0.613)	0.199	B
L64	Rochers du Basto	30	10	14.66	0.145 (0.113–0.184)	0.063 (0.014)	0.116 (0.025)	0.574 (0.371–0.630)	0.185	B

Pop. code: population code; Est. pop. size: estimated population size; $\%P_{pop}$: percentage of polymorphic markers; H_s: Bayesian estimate of within population gene diversity; $H_{e\ all}$: within population gene diversity assuming Hardy-Weinberg (H-W) equilibrium and using all 116 AFLP markers; $H_{e\ only\ polym.}$: within population gene diversity assuming H-W equilibrium and using the polymorphic 63 AFLP markers; F: the probability that two randomly chosen genes have a common ancestor in the particular population; M: average number of migrants received per generation; HPD: highest posterior density interval; SE: standard error of the mean.

G generations (G = number of generations elapsed since immigration), individuals were split into two groups based on their population of origin (group A or group B populations) and this membership vector was used as prior population information in the assignment analysis (Falush & al., 2003). The analysis was carried out using a wide range of υ (0.01, 0.1, 0.3) as recommended in Pritchard & al. (2000). To infer individuals with relatively recent immigrant origin, G was set to 1.

To assess whether the genetic structure of *S. florulenta* populations corresponds to a pure drift or a drift-migration model, the algorithm implemented in the software 2mod was applied (Ciofi & al., 1999). To estimate the relative likelihood of the two models and F for each population (the probability that the first event among two randomly chosen alleles in a particular population is a coalescent rather than a migration or founder event) 10^5 iterations with a 10^4 iterations burn-in period were used. Each analysis was repeated three times to confirm the convergence of the Markov chain. Under the drift-migration model, the proportion of migrants received per generation (M) can be calculated once F is known [M = (1 – F) / 4F], thus the contribution of drift relative to migration can be assessed in each population.

AFLP data were further investigated with Principal Coordinates Analysis (PCoA; Podani, 2000a) using the Jaccard index (Podani, 2000a) with the SYNTAX 2000 software (Podani, 2000b) for comparison with the results of Bayesian clustering.

To investigate whether individual populations or groups of populations are genetically differentiated, an exact test of population differentiation (Raymond & Rousset, 1995) was conducted in TFPGA-v1.3 (Miller, 1997) with allele frequencies estimated using the method of Lynch & Milligan (1994) assuming H-W equilibrium. The significance of each cross tabulation was checked using an MCMC algorithm with default settings.

The number of polymorphic markers and private alleles were calculated for each population separately. We used the Bayesian method of Holsinger & al. (2002) to estimate θ^B (an analogue of F_{st}) and within-population expected heterozygosities (H_s). The software HICKORY-v1.0.4 (Holsinger & al., 2002) was applied with a non-informative prior on F_{is} (within-population inbreeding coefficient) using the f free model. This model was favored over the f = 0, θ = 0 and full models according to the Deviance Information Criterion (DIC 1,693.9, 1,728.7, 2,880.2 and 1,728.0, respectively). To check the consistency of the results, five runs were conducted with the default parameter values. F_{st} was also estimated assuming total outbreeding and inbreeding using POPGENE-v1.32 (Yeh & al., 1997).

Variation among and within groups was investigated by analyses of molecular variance (AMOVA; Excoffier & al., 1992) performed on the total dataset and on each population group defined by the Bayesian and multivariate analyses. All calculations were performed with GenAlEx 6.0 (Peakall & Smouse, 2005) and significance levels were derived from 1,000 permutations.

To test whether populations show a regional drift-migration equilibrium, correlation between genetic and geographic distance matrices was assessed (Hutchison & Templeton, 1999). Geographic distances between populations were calculated using the 'Haversine' formula (Sinnott, 1994). Genetic distances among populations were calculated as pairwise θ^B (F_{st}) values applying the f free model of HICKORY-v1.0.4 (Holsinger & al., 2002). Mantel correlations were calculated among the genetic and geographical distance matrices and their significance was tested using 1,000 random permutations in ARLEQUIN-v3.11 (Excoffier & al., 2005).

RESULTS

Using four primer combinations, 116 unambiguously scorable markers were generated, ranging in length from 90 to 300 bp (data not shown). Sixty-three (54.31%) markers were polymorphic, the number of polymorphic markers per primer combination ranging from 9 to 22, with an average of 15.75 (Table 2). The 168 individual samples produced 158 different AFLP banding patterns. None of the 15 populations was monomorphic, nor did any of the populations or population groups (A and B; see below) present any private markers (i.e., fragments present in one population only).

Population genetic differentiation. — The modal value of ΔK obtained by Bayesian clustering analysis indicated that the most likely number of ancestral gene pools is 2 (Fig. 2). Although there was also evidence for population structure at K = 5 and 9, K = 2 is the most probable solution for the following reasons. First, the high modal value at K = 2 indicates a strong signal, thus K = 2 obviously describes the main structure of the dataset (Evanno & al., 2005). Second, when using the model of correlated allele frequencies, STRUCTURE usually overestimates

Table 2. Total number of scorable fragments and percent of polymorphism for the four primer combinations used in the study.

Primer pairs	Total fragments	Polymorphic fragments	% polymorphism
E-ATC/M-CTC	33	15	45.45
E-AAA/M-CTC	32	22	68.75
E-ATG/M-CAG	25	9	36.00
E-AGT/M-CTA	26	17	65.38
Total	116	63	54.31

the number of ancestral gene pools (Pritchard & al., 2000; Falush & al., 2003). Finally, solution at K = 2 resolved two well interpretable and structured groups of populations, while solutions at K = 5 and 9 revealed a continuous cline in the proportional membership of individuals (data not shown). Accordingly, populations were assigned to two groups: group A and B (Fig. 1B–D). Group A is formed by populations L36, L38, L39, L32, L26, L18, L47, L24, and L31, whereas Group B is formed by populations L6, L53, L58, L57, L64, and L51. Populations assigned to group A occur across the whole distributional range of the species, while populations assigned to group B occur exclusively in the Vésubie/Roya Region (Fig. 1B, C).

The first three axes of the PCoA explained approximately 43% of the variation of the data (Fig. 3) and the first axis identified the same two groups of populations as the Bayesian analysis (groups A and B; Fig. 1B). However, the two point clouds were not sharply separated on the plot and heterogeneity was greater within than between groups.

In line with the results of the Bayesian clustering analysis, the exact test of population differentiation indicated that populations of group A were significantly differentiated from populations of group B ($\chi^2 = 163.55$, $P < 0.05$; Raymond & Rousset, 1995). Significant pairwise differences between population allele frequencies were only found when comparing populations of the two groups (Table 3). Indeed, none of the within-group comparisons was significant (Table 3).

The Bayesian estimate of genetic differentiation among all populations ($\theta^B = 0.257$, 95% Highest Posterior Density [HPD] interval: 0.217–0.297) was slightly lower than estimates assuming obligate outbreeding ($F_{st} = 0.356$) or inbreeding ($F_{st} = 0.351$). Genetic differentiation among populations within groups A and B obtained by Bayesian analysis was slightly lower than between group estimates ($\theta^B = 0.114$, 95% HPD: 0.083–0.149 and $\theta^B = 0.136$, 95% HPD: 0.086–0.200, respectively).

Population genetic diversity. — The percentage of intra-population polymorphic markers (hereafter referred to as $\%P_{pop}$) ranged from 10.34% to 28.45% with a mean of 19.08% (Table 1). Bayesian estimates of within-population gene diversities (H_s) ranged from 0.108 to 0.181 (Table 1). The percentage of polymorphic markers ($\%P_{pop}$) and the Bayesian estimates of H_s were significantly correlated (Spearman R = 0.837, $P < 0.001$). The percentage of polymorphic markers ($\%P_{pop}$) was significantly higher in populations of group A than group B (Mann-Whitney U = 6.5; $P < 0.01$). By contrast, there was no significant difference in Bayesian estimates of H_s between the two groups (Mann-Whitney U = 24.00, $P = 0.776$) but group A populations in the Tinée region tended to show higher gene diversities than group A populations located outside of this area (Mann-Whitney U = 2.00, $P = 0.06$).

A high proportion of the total molecular variance was partitioned within populations (59%), while smaller but considerable proportions were found between the two groups defined by the Bayesian and PCoA analyses (31%; Table 4). By contrast, molecular variance among populations within groups A and B were low (10%; Table 4). The amount of intra-population genetic variation increased to 87% and 86%, respectively, when populations of group A and B were considered separately (Table 4).

Current rate and direction of migration. — In the assignment analysis, 163 of the 168 plants were correctly assigned to their subgroup (group A or B) of origin (with 0.8 or higher probability), regardless of which value was chosen for parameter υ (the probability that an individual is an immigrant; data not shown). Indeed, only five

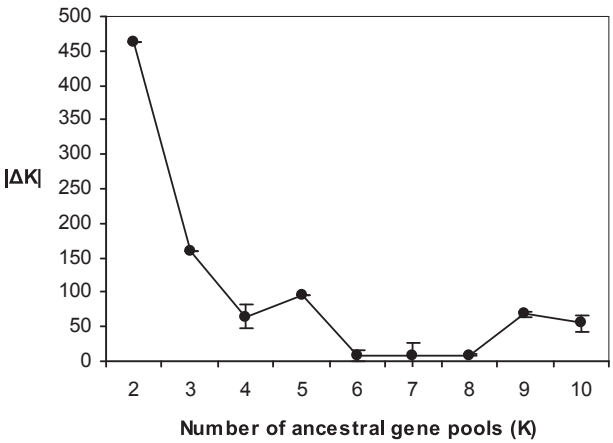

Fig. 2 Absolute change in the ln likelihood of the data (|ΔK| = |ln Pr(X|K) – ln Pr(X|K – 1)|) as a function of the number of ancestral gene pools (K) resulting from the Bayesian clustering analysis. Mean values and their standard deviations are shown over the ten independent runs conducted.

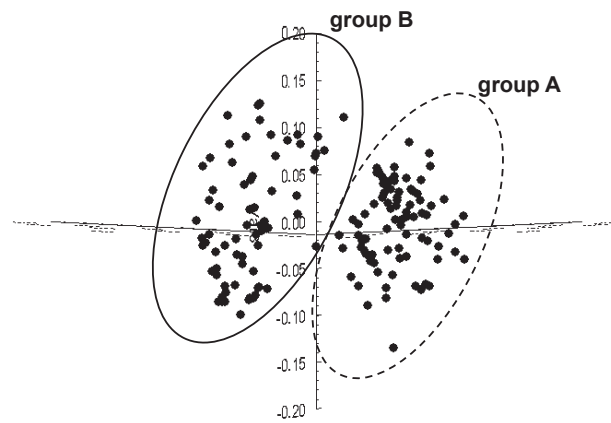

Fig. 3. Results of the principal coordinate analysis (PCoA). Individuals belonging to populations assigned to group A or to group B are shown in ellipses. The first three axes of the PCoA explained 43% of the total variation (21%, 16% and 6% respectively).

individuals were judged to have immigrant origins, because they had low posterior probabilities of belonging to their subgroup (0.1 or lower) but relatively high posterior probabilities of being immigrants (0.9 or more). Posterior probabilities that they are themselves immigrants were lower (0.22–0.33) than the probability that one of their parents were immigrants (0.40–0.66; data not shown). Individuals of immigrant origin were only detected in group A, suggesting a biased pattern of migration (Fig. 1D). Based on this proportion of recent immigrants (5/168 = 0.030), and assuming equally probable bidirectional migration and the harmonic mean (55.38; Table 1) of population sizes as effective number of individuals, we finally estimated the number of migrants per generation (Nm) between group A and B as Nm = 1.661/2 = 0.8307.

The genetic data unequivocally supported the migration-drift equilibrium model of 2mod with $P = 1.00$. F values (the probability that two alleles share a common ancestor in a particular population) were on average higher in group B than in group A populations (0.264 and 0.523 respectively; Table 1). On average, group A populations received more immigrants per generation (M) than group B populations (0.802 and 0.260 respectively; Table 1). These differences were significant based on a Mann-Whitney U-test ($P = 0.0047$).

The medians of the pairwise θ^{B} values estimated by the f free model of the Bayesian approach were 0.106 (quartiles: 0.072–0.160) and 0.157 (quartiles: 0.090–0.388) for populations of group A and group B, respectively. There was no correlation among geographic and genetic

Table 3. Significance values of exact tests of population differentiation using allele frequencies.

Populations	L36	L38	L39	L32	L26	L18	L47	L24	L31	*L6*	*L53*	*L58*	*L51*	*L57*	*L64*
L36	0														
L38	1.000	0													
L39	1.000	1.000	0												
L32	0.955	0.998	1.000	0											
L26	1.000	0.997	1.000	0.774	0										
L18	1.000	1.000	1.000	1.000	1.000	0									
L47	1.000	1.000	1.000	0.997	1.000	1.000	0								
L24	0.749	0.257	0.997	0.843	0.415	1.000	0.971	0							
L31	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.216	0						
L6	0.231	0.953	0.080	0.386	**0.003**	0.651	**0.022**	0.146	0.110	0					
L53	0.070	0.707	0.757	0.400	0.183	0.837	**0.006**	**0.023**	0.434	1.000	0				
L58	**0.000**	**0.011**	**0.001**	0.152	**0.000**	0.253	**0.000**	**0.024**	**0.000**	1.000	1.000	0			
L51	**0.000**	**0.012**	**0.000**	**0.001**	**0.000**	0.403	**0.000**	0.271	**0.008**	0.997	1.000	1.000	0		
L57	**0.000**	**0.003**	**0.000**	**0.000**	**0.000**	**0.004**	**0.000**	0.074	**0.000**	1.000	0.999	1.000	1.000	0	
L64	**0.021**	0.385	0.562	0.225	**0.019**	0.751	**0.002**	0.150	**0.034**	1.000	1.000	1.000	1.000	1.000	0

Results of between-group comparisons are framed and significant comparisons ($P < 0.05$) are shown in bold face. Codes of group B populations are shown in italics.

Table 4. Summary table of the analysis of molecular variance (AMOVA) for *Saxifraga florulenta*.

	Source of variance	d.f.	Sum of squares	Mean of squared deviation	Variance component	% total variance	Fixation index
Total	Between groups	1	158.585	158.585	1.838	31%	Φrt = 0.3142*
	Among populations within groups	13	125.752	9.673	0.557	10%	Φpr = 0.1388*
	Within populations	153	528.603	3.455	3.455	59%	Φpt = 0.4094*
Group A	Among populations	8	87.234	10.904	0.634	14%	Φpt = 0.1403*
	Within populations	91	353.386	3.883	3.883	86%	
Group B	Among populations	5	38.518	7.704	0.431	13%	Φpt = 0.1324*
	Within populations	62	175.217	2.826	2.826	87%	

* = $P \leq 0.001$. Levels of significance are based on 1,000 permutations.

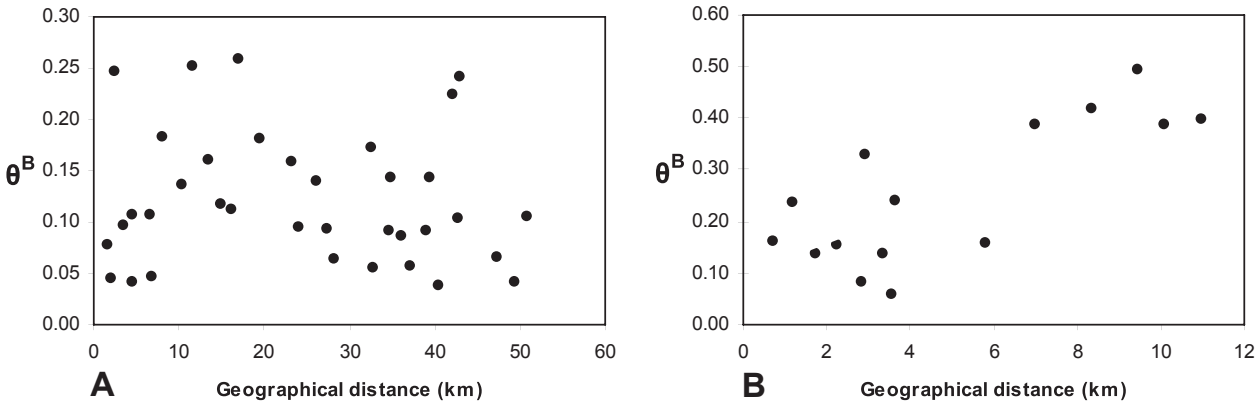

Fig. 4. Correlation of geographical and genetic distances across populations assigned to group A (A) and B (B), respectively. Pairwise θ^B values were estimated using the f free model of HICKORY-v.1.0.4 (Holsinger & al., 2002).

distance matrices across all populations (r_m = 0.069, *P* = 0.223) and no correlation was found within group A populations either (Fig. 4; r_m = –0.107, *P* = 0.240). By contrast, group B populations showed moderate but not significant correlation between geographic and genetic distances (Fig. 4; r_m = 0.421, *P* = 0.112).

DISCUSSION

The remarkable morphological distinctiveness of *Saxifraga florulenta*, its unusual life history, diploid chromosome number, isolated taxonomic position, strict adaptation to siliceous substrates, and restricted distribution within the Maritime Alps (Webb & Gornall, 1989; Grey-Wilson, 1985; Conti & al., 1999, Filipello & Gardini-Peccenini, 1985) induced several botanists to suggest that it originated in the Tertiary and survived the Pleistocene climatic rigors in refugia (Ozenda, 1950; Pawlowski, 1970; Martini, 1982, 1992; Médail & Verlaque, 1997). The present study based on AFLP provides additional evidence that is consistent with the proposed relictual origin of this species, while contributing novel information about the role of local refugia in the survival of silicicolous plants in the Alps. Altogether, the results discussed below can be useful for the long-term conservation of *S. florulenta*.

***Saxifraga florulenta* likely survived the glacial maxima in one main refugium of the Maritime Alps.** — During the Pleistocene climatic cycles, some Tertiary taxa escaped extinction by finding refuge in areas that remained unglaciated even during glacial maxima (Bennett & al., 1991; Lang, 1994; Ehlers & Gibbard, 2004; Comes & Kadereit, 1998; Stehlik, 2003; Tribsch & Schönswetter, 2003; Schönswetter & al., 2005). The changes in species ranges associated with glacial advancement and retreat likely left a signature on the genetic structure of Alpine populations (Hewitt, 1996, 2000; Comes & Kadereit, 1998; Tribsch & Schönswetter, 2003). In situ survival in refugia at the rear edge of the distribution range is expected to be associated with genetic bottlenecks and small population sizes, which may be reflected by contemporary low within-population genetic diversity (Hewitt, 1996, 2000; Hampe & Petit, 2005). In contrast, restricted gene flow among refugial populations leads to inter-population genetic differentiation resulting in well defined refugial gene pools and high levels of regional diversity in the refugial area (Comes & Kadereit, 1998; Petit & al., 2003; Hampe & Petit, 2005). However, if survival in different refugia happened to be associated with cycles of altitudinal/latitudinal displacement, genetic diversity may be severely reduced globally (Hampe & Petit, 2005).

The narrow endemic *S. florulenta* is characterised by a level of species-wide AFLP (%P = 54.31; Table 2) and within population genetic diversity (H_s = 0.108–0.181, $H_{e\ only\ polym.}$ = 0.060–0.211) that fall at the lower end of values recorded so far in natural populations of plant species (Tero & al., 2003; Schönswetter & al., 2004a, 2006a; Kang & al., 2005; Gaudeul, 2006; Jacquemyn & al., 2006; Liu & al., 2006). Comparably low values of %P and average within-population heterozygosities (H_e) have been found in endangered or rare species with narrow distribution, for instance, %P = 42.6% in *Sticherus flabellatus* (Keiper & McConchie, 2000), %P = 24%, H_e = 0.002–0.056 in *Hypochaeris acaulis* (Tremetsberger & al., 2003), H_e = 0.035–0.143 in *Medicago citrina* (Juan & al., 2004), H_e = 0.000–0.053 in *Carex atrofusca* (Schönswetter & al., 2006b), %P = 53%, H_e = 0.16–0.19 in *Femeniasia balearica* (Vilatersana & al., 2007).

The low levels of species-wide and within-population genetic diversity detected in *S. florulenta* are congruent with the proposed hypothesis of its relictual origin and in situ survival. In situ persistence over numerous climatic cycles may have been only possible by altitudinal shifts of the species' distribution to match appropriate climatic

conditions. This process has been likely supported by the heterogeneous topography and high diversity of microhabitats per surface area of the Maritime Alps (Casazza & al., 2005). Repeated altitudinal shifts may have led to the genetic depauperation of the species as inferred in other case studies of Alpine plants (Schönswetter & al., 2003, 2006a,b).

Integrating geological and palaeo-climatological data with the current distribution of *S. florulenta* indicates that ice-free nunataks in the Tinée and Vésubie/Roya regions might have served as potential refugia during the Pleistocene glaciations (Fig. 1B; Filipello & Gardini-Peccenini, 1985; Webb & Gornall, 1989; Diadema & al., 2005; M. Dubar, CNRS, France, unpub. res.). Importantly, these regions are composed of siliceous bedrock, enabling the survival of edaphically specialised, high-altitude species such as *S. florulenta* during the Pleistocene glacial maxima. Furthermore, the Tinée valley has been recently proposed as a local refugium based on its high number of endemics (G. Casazza, DIP.TE.RIS, University of Genova, Italy, unpub. res.). The genetic pattern detected in our study supports the existence of two ancestral gene pools within *S. florulenta*, in agreement with a period of isolation (Figs. 1B, 3; Table 3), a pattern seemingly congruent with the survival of *S. florulenta* in two main refugial areas (i.e., the Tinée valley and the Vésubie/Roya region), followed by a period of isolation. However, the relatively low levels of molecular variance and genetic differentiation between the two population groups suggest that their split is more recent than expected under the strict two-refugia hypothesis, which assumes several thousand years of isolation. Furthermore, the rather weak separation of the two population groups on the PCoA plot and the lack of group specific AFLP markers raise the possibility that group B populations originated from the widespread group A populations.

Group A populations occur across the entire distributional range of *S. florulenta* (Fig. 1B, C). Hypothesising that gene pool A was maintained in the unglaciated nunataks of the Tinée valley implies that group A populations of the Argentera and Vésubie/Roya regions represent descendants of an original refugial population. Accordingly, level of within-population genetic diversity (H_s) of group A populations was lower in the Argentera and Vésubie/Roya regions than in the Tinée valley, suggesting successive eastwards colonisation events from the refugial gene pool of the Tinée valley (Fig. 1C). To sum up, the genetic data appear to support the existence of two ancestral gene pools in agreement with palaeo-climatological and geological data. However, the relatively low level of genetic differentiation between the two groups of populations representing the two gene pools partly contradicts the hypothesis of two refugia during the Pleistocene glaciations. Hence, it is more likely that Pleistocene survival of *S. florulenta* populations took place in the Tinée valley and separation of group A and B populations occurred after the recolonisation of the Argentera and the Vésubie/Roya regions. However, to rigorously test whether separation of the two gene pools occurred before or after the Pleistocene glaciations the depth of divergence between the two gene pools needs to be estimated using additional molecular markers with well-known mutational processes.

Current rate and direction of among-population migration. — In this study two algorithms are used to calculate recent migration rates. Estimates of the number of migrants per generation in *S. florulenta* vary between the assignment test and the coalescent analyses (Nm = 0.825, M = 0.802 and 0.260 for group A and B, respectively), one reason being that the harmonic mean of the census population sizes overestimates the effective number of individuals. All our estimates are though comparable with migration rates calculated for other plant species with genetically well-differentiated populations. For instance, estimates of the number of migrants received per generation varied between 0.135 and 1.451 in populations of *Myricaria laxiflora*, an endemic to the Yangtze River valley (Liu & al., 2006), while slightly lower values (0.133–0.677) were observed in populations of the endangered *Silene tatarica* (Tero & al., 2003). The average low estimates for recent migration rates in *S. florulenta* further indicate that migration may not be sufficient to balance the effect of genetic drift (Wright, 1931; Wang, 2004). However, it should be kept in mind that migration rates may vary from year to year and our estimates only provide a snapshot of migration events over the past two to three generations.

Assuming no difference in dispersal ability between group A and B populations and no directional preference of dispersal, equal amounts of migrants would be expected in both directions. However, migration between the two groups of populations inferred by the assignment approach (Pritchard & al., 2000) and the Bayesian-coalescent simulation of 2mod (Ciofi & al., 1999) turned out to be strongly unidirectional with most of the migrants received by group A (Fig. 1D; Table 1). The inferred pattern of migration may be caused either by unknown historical or contemporary processes acting on natural populations or by sampling bias for fewer individuals were genotyped from group B than from group A populations, increasing the likelihood of undetected rare immigrants in the former. Our current genetic data appear to be insufficient to distinguish among the alternative hypotheses for the inferred patterns of migration.

A recently established group of populations originating from a homogeneous genetic pool is expected to show no considerable genetic structure (Hutchinson & Templeton, 1999). Subsequently, pairwise F_{st} values and geographic distances should not be correlated (Hutchinson

& Templeton, 1999). Afterwards, if migration among populations remains dominant compared to drift, no correlation should develop between geographic and genetic distances. By contrast, if the effect of drift is stronger than that of migration, genetic and geographic distances should become correlated.

In *S. florulenta*, populations of group A showed no correlation among genetic and geographic distances (Fig. 4). High within-group genetic homogeneity suggests either frequent within-group gene flow and/or recent establishment of most of the populations compared to the marker's mutation rates. Since recent migration rates among populations of *S. florulenta* appear to be low (see previous paragraph), the lack of correlation between genetic and geographic distances indicates that the amount of migration since establishment has been insufficient to attain regional equilibrium (Hutchinson & Templeton, 1999). Similarly, a moderate, but non-significant correlation was observed among populations of group B (Figs. 1, 4).

CONCLUSIONS

The present results concerning the genetic structure of *S. florulenta* have important implications for its long-term in situ survival. The low level of species-wide genetic diversity in addition to small population sizes and low levels of migration represent a threat for the survival of *S. florulenta* (Wright, 1931; Wang, 2004), because this species is susceptible to extinction due to genetic and demographic processes (Ellstrand & Elam, 1993; Frankham, 1999; Reed & Frankham, 2003; Raffl & al., 2006). Indeed, the low migration rates estimated for *S. florulenta* may not be sufficient to balance the effect of genetic drift (Wright, 1931; Wang, 2004), implying that further fragmentation of populations should be avoided and that both gene pools should be protected to preserve and maintain most of the genetic variability of the species (Petit & al., 1998).

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LITERATURE CITED

Barbero, M. 1967. L'endémisme dans les Alpes Maritimes et Ligures. *Bull. Soc. Bot. France* 114: 179–199.

Bennett, K.D., Tedzakis, P.C. & Willis, K.J. 1991. Quaternary refugia of North European trees. *J. Biogeogr.* 18: 103–115.

Bland, B. 2000. *Silver Saxifrages. A Guide to Encrusted Saxifrages for Gardeners and Botanists*. Alpine Garden Society Publications Limited, Worcestershire.

Casazza, G., Barberis, G. & Minuto, L. 2005. Ecological characteristics and rarity of endemic plants of the Italian Maritime Alps. *Biol. Cons.* 123: 361–371.

Casazza, G., Zappa, E., Mariotti, M.G., Médail, F. & Minuto, L. 2008. Ecological and historical factors affecting distribution pattern and richness of endemic plant species: the case of the Maritime and Ligurian Alps hotspot. *Div. Distrib.* 14: 47–58.

Ciofi, C., Beaumont, M.A., Swingland, I.R. & Bruford, M.W. 1999. Genetic divergence and units for conservation in the Komodo dragon *Varanus komodoensis*. *Proc. Roy. Soc. London, Ser. B, Biol. Sci.* 266: 2269–2274.

Comes, H.P. & Kadereit, J.W. 1998. The effect of Quaternary climatic changes on plant distribution and evolution. *Trends Pl. Sci.* 3: 432–438.

Comes, H.P. & Kadereit, J.W. 2003. Spatial and temporal patterns in the evolution of the flora of the European Alpine System. *Taxon* 52: 451–462.

Conti, E., Soltis, D.E., Hardig, T.M. & Schneider, J. 1999. Phylogenetic relationships of the silver saxifrages (*Saxifraga*, sect. *Ligulatae* Haworth): implications for the evolution of substrate specificity, life histories, and biogeography. *Molec. Phylog. Evol.* 13: 536–555.

Reed, D.H. & Frankham, R. 2003. Correlation between fitness and genetic diversity. *Cons. Biol.* 17: 230–237.

Diadema, K., Bretagnolle, F., Affre, L., Yuan, Y.-M. & Médail, F. 2005. Geographic structure of molecular variation of *Gentiana ligustica* (Gentianaceae) in the Maritime and Ligurian regional hotspot, inferred from ITS sequences. *Taxon* 54: 887–894.

Ehlers, J. & Gibbard, P.L. 2004. *Quaternary Glaciations Extent and Chronology*, part 1, *Europe*. Elsevier, Oxford.

Ellstrand, N.C. & Elam, D.R. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual Rev. Ecol. Syst.* 24: 217–242.

Evanno, G., Regnaut, S. & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molec. Ecol.* 14: 1611–2620.

Excoffier, L., Laval, G. & Schneider, S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinform. Online* 1: 47–50.

Excoffier, L., Smouse, P.E. & Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restrictions sites. *Genetics* 131: 479–491.

Falush, D., Stephens, M. & Pritchard, J.K. 2003. Inference of population structure: extensions to linked loci and correlated allele frequencies. *Genetics* 164: 1567–1587.

Falush, D., Stephens, M. & Pritchard, J.K. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molec. Ecol. Notes* 7: 574–578.

Filipello, S. & Gardini-Peccenini, S. 1985. The Italian Peninsular and Alpine regions. Pp. 71–88 in: Gómez-Campo, C. (ed.), *Plant Conservation in the Mediterranean Area*. Junk, Dordrecht.

Focquet, P. & Romain, J. 1988. La Saxifrage florulente. Une plante, un symbole, une légende. *Rivièra Sci.* 10: 25–39.

Frankham, R. 1999. Resolving conceptual issues in conservation genetics: the roles of laboratory species and meta-analyses. *Hereditas* 130: 195–201.

Gaudeul, M. 2006. Disjunct distribution of *Hypericum nummularium* L. (Hypericaceae): molecular data suggest bidirectional colonization from a single refugium rather than survival in distinct refugia. *Biol. J. Linn. Soc.* 87: 437–447.

Gaudeul, M., Taberlet, P. & Till-Bottraud, I. 2000. Genetic diversity in an endangered alpine plant, *Eryngium alpinum* L. (Apiaceae), inferred from amplified fragment length polymorphism markers. *Molec. Ecol.* 9: 1625–1637.

Grey-Wilson, C. 1985. Plants in peril, *Saxifraga florulenta*. *Kew Mag.* 2: 232–234.

Hampe, A. & Petit, R.J. 2005. Conserving biodiversity under climate change: the rear edge matters. *Ecol. Lett.* 8: 461–467.

Hewitt, G.M. 1996. Some genetic consequences of the ice ages and their role in divergence and speciation. *Biol. J. Linn. Soc.* 58: 247–276.

Hewitt, G.M. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405: 907–913.

Holderegger, R. 1996. Reproduction of the rare monocarpic species *Saxifraga mutata* L. *Bot. J. Linn. Soc.* 122: 301–313.

Holderegger, R., Stehlik, I. & Abbott, R.J. 2002. Molecular analysis of the Pleistocene history of *Saxifraga oppositifolia* in the Alps. *Molec. Ecol.* 11: 1409–1418.

Holsinger, K.E., Lewis, P.O. & Dey, D.K. 2002. A Bayesian approach to inferring population structure from dominant markers. *Molec. Ecol.* 11: 1157–1164.

Hughes, P.D., Woodward, J.C. & Gibbard, P.L. 2006. Quaternary glacial history of the Mediterranean mountains. *Progr. Physical Geogr.* 30: 334–364.

Hutchison D.W. & Templeton A.R. 1999. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution* 53: 1898–1914.

Jacquemyn, H., Honnay, O., Van Looy, K. & Breyne, P. 2006. Spatiotemporal structure of genetic variation of a spreading plant metapopulation on dynamic riverbanks along the Meuse River. *Heredity* 96: 471–478.

Juan, A., Crespo, M.B., Cowan, R.S., Lexer, C. & Fay, M.F. 2004. Patterns of variability and gene flow in *Medicago citrina*, an endangered endemic of islands in the western Mediterranean, as revealed by amplified fragment length polymorphism (AFLP). *Molec. Ecol.* 13: 2679–2690.

Kang, M., Ye, Q.G. & Huang, H.W. 2005. Genetic consequence of restricted habitat and population decline in endangered Isoetes sinensis (Isoetaceae). *Ann. Bot. (London)* 96: 1265–1274.

Keiper, F.J. & McConchie, R. 2000. An analysis of genetic variation in natural populations of *Stricherus flabellatus* R. Br. (St John) using amplified fragment length polymorphism (AFLP) markers. *Molec. Ecol.* 9: 571–581.

Lang, G. 1994. *Quartäre Vegetationsgeschichte Europas*. Gustav Fischer Verlag, Jena.

Liu, Y.F., Wang, Y. & Huang, H.W. 2006. High interpopulation genetic differentiation and unidirectional linear migration patterns in *Myricaria laxiflora* (Tamaricaceae), an endemic riparian plant in the Three Gorges valley of the Yangtze River. *Amer. J. Bot.* 93: 206–215.

Lynch, M. & Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. *Molec. Ecol.* 3: 91–99.

Martini, E. 1982. Lineamenti geobotanici delle Alpi Liguri e Marittime: endemismi e fitocenosi. *Lav. Soc. Ital. Biogeogr.* 9: 51–134.

Martini, E. 1992. Note critiche sulle fitocenosi di casmofite individuate nelle Alpi Marittime e corologia di specie fitogeograficamente significative. *Biogeographia* 16: 55–89.

Médail, F. & Quézel, P. 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. *Ann. Missouri Bot. Gard.* 84: 112–127.

Médail, F. & Verlaque, P. 1997. Ecological characteristics and rarity of endemic plants from southeast France and Corsica: implications for biodiversity conservation. *Biol. Cons.* 80: 269–281.

Miller, M.P. 1997. *Tools For Population Genetic Analyses (TFPGA): A Windows (TM) Program for the Analysis of Allozyme and Molecular Population Genetic Data*. Computer software distributed by the author.

Minuto, L., Grassi, F. & Casazza, G. 2006. Ecogeographic and genetic evaluation of endemic species in the Maritime Alps: the case of *Moheringia lebrunii* and *M. sedoides* (Caryophyllaceae). *Pl. Biosystems* 140: 146–155.

Ozenda, P. 1950. Eléments géographiques et endémisme dans les Alpes Maritimes et Ligures. *Bull. Soc. Bot. France* 97: 141–156.

Pawlowski, B. 1970. Remarques sur l'endémisme dans la flore des Alpes et des Carpates. *Vegetatio* 21: 181–243.

Peakall, R. & Smouse, P. 2005. *GenAlEx 6: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research*. http://www.anu.edu.au/(BoZo/GenAlEx/. The Australian National University, Canberra.

Petit, R.J., Mousadik, A.E. & Pons, O. 1998. Identifying populations for conservation on the basis of genetic markers. *Cons. Biol.* 12: 844–855.

Petit, R.J., Aguinagalde, I., de Beaulieu, J-L., Bittkau, C., Brewer, S., Cheddadi, R., Ennos, R., Fineschi, S., Grivet, D., Lascoux, M., Mohanty, A., Müller-Starck, G., Demesure-Musch, B., Palmé, A., Martín, J.P., Rendell, S., Vendramin, G.G. 2003. Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* 300: 1563–1565.

Podani, J. 2000a: *Introduction to the Exploration of Multivariate Biological Data*. Backhuys, Leiden.

Podani, J. 2000b. *SYN-TAX 2000, Data Analysis in Ecology and Systematics, Users Manual*. Sciencia Publishing, Budapest.

Pritchard, J.K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

Raffl, C., Schönswetter, P. & Erschbamer, B. 2006. 'Saxsess'—genetics of primary succession in a pioneer species on two parallel glacier forelands. *Molec. Ecol.* 15: 2433–2440.

Raymond, M.L. & Rousset F. 1995. An exact test for population differentiation. *Evolution* 49: 1280–1283.

Schönswetter, P., Paun, O., Tribsch, A. & Niklfeld, H. 2003. Out of the Alps: colonization of Northern Europe by East

Alpine populations of the Glacier Buttercup *Ranunculus glacialis* L. (Ranunculaceae). *Molec. Ecol.* 12: 3373–3381.

Schönswetter, P., Popp, M. & Brochmann, C. 2006a. Rare arctic-alpine plants of the European Alps have different immigration histories: the snow bed species *Minuartia biflora* and *Ranunculus pygmaeus*. *Molec. Ecol.* 15: 709–720.

Schönswetter, P., Popp, M. & Brochmann, C. 2006b. Central Asian origin of and strong genetic differentiation among populations of the rare and disjunct *Carex atrofusca* (Cyperaceae) in the Alps. *J. Biogeogr.* 33: 948–956.

Schönswetter, P., Stehlik, I., Holderegger, R. & Tribsch, A. 2005. Molecular evidence for glacial refugia of mountain plants in the European Alps. *Molec. Ecol.* 14: 3547–3555.

Schönswetter, P., Tribsch, A. & Niklfeld, H. 2004a. Amplified Fragment Length Polymorphism (AFLP) suggest old and recent immigration into the Alps by the artic-alpine annual *Comastoma tenellum* (Gentianaceae). *J. Biogeogr.* 31: 1673–1681.

Schönswetter, P., Tribsch, A., Stehlik, I. & Niklfeld, H. 2004b. Glacial history of high alpine *Ranunculus glacialis* (Ranunculaceae) in the European Alps in a comparative phylogeographical context. *Biol. J. Linn. Soc.* 81: 183–195.

Sinnott, R.W. 1984. Virtues of the Haversine. *Sky and Telescope* 68: 159–159.

Stehlik, I. 2003. Resistance or emigration? Response of alpine plants to the ice ages. *Taxon* 52: 499–510.

Stehlik, I., Blattner, F.R., Holderegger, R. & Bachmann, K. 2002a. Multiple glacial nunatak survival of the high alpine *Eritrichium nanum* (L.) Gaudin within the European Alps. *Molec. Ecol.* 11: 2027–2036.

Stehlik, I., Schneller, J.J. & Bachmann, K. 2002b. Immigration and in situ glacial survival of the low-alpine *Erinus alpinus* (Scrophulariaceae). *Biol. J. Linn. Soc.* 77: 87–103.

Taberlet, P. & Cheddadi, R. 2002. Quaternary refugia and persistence of biodiversity. *Science* 297: 2009–2010.

Tero, N., Aspi, J., Siikamaki, P., Jakalaniemi, A. & Tuomi, J. 2003. Genetic structure and gene flow in a metapopulation of an endangered plant species, *Silene tatarica*. *Molec. Ecol.* 12: 2073–2085.

Tremetsberger, K., Stuessy, T.F., Guo, Y.P., Baeza, C.M., Weiss, H. & Samuell R.B. 2003. Amplified fragment length polymorphism (AFLP) variation within and among populations of *Hypochaeris acaulis* (Asteraceae) of Andean southern South America. *Taxon* 52: 237–245.

Tribsch, A. & Schönswetter, P. 2003. Patterns of endemism and comparative phylogeography confirm palaeoenvironmental evidence for Pleistocene refugia in the Eastern Alps. *Taxon* 52: 477–497.

Tribsch, A., Schönswetter, P. & Stuessy, T. 2002. *Saponaria pumila* (Caryophyllaceae) and the ice-age in the European Alps. *Amer. J. Bot.* 89: 2024–2033.

Yeh, F.C., Yang, R.C., Boyle, T.B.J., Ye, Z.H. & Mao, J.X. 1997. *POPGENE, The User-Friendly Shareware for Population Genetic Analysis.* Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton.

Vilatersana, R., Susanna, A. & Brochmann, C. 2007. Genetic variation in *Femeniasia* (Compositae, Cardueae), an endemic and endangered monotypic genus from the Balearic Islands (Spain). *Bot. J. Linn. Soc.* 153: 97–107.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407–4414.

Walter, K.S. & Gillett, H.J. (eds.). 1998. *1997 IUCN Red List of Threatened Plants.* Compiled by the World Conservation Monitoring Centre. IUCN – The World Conservation Union, Gland, Switzerland and Cambridge, U.K.

Wang, J. 2004. Application of the one-migrant-per-generation rule to conservation and management. *Cons. Biol.* 18: 332–343.

Webb, D.A. & Gornall, R.J. 1989. *Saxifrages of Europe*. Christopher Helm, London.

Wright, S. 1931. Evolution in Mendelian populations. *Genetics* 16: 97–257.