Isolated populations of a rare alpine plant show high genetic diversity and considerable population differentiation

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INTRODUCTION

Alpine landscapes are characterized by pronounced environmental gradients and heterogeneous topography (Körner, 2003). Steep valleys and high mountain ridges demarcate the plants’ habitats and might consequently limit gene flow among plant populations and colonization opportunities of new sites (Cain et al., 2000; Theurillat and Guisan, 2001).

Limited gene flow due to habitat isolation could lead to a stronger genetic differentiation among populations compared with plants found in less-isolated habitats and/or lower altitudes (Till-Bottraud and Gaudeul, 2002). Genetic diversity and differentiation can also be influenced by several other factors such as population size, founder effects and mating system. For example, genetic drift can be enhanced in small populations, which could lead to extinction of alleles and loss of genetic variability (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Young et al., 1996; Lowe et al., 2004).

Genetic diversity may be reduced as a consequence of a bottleneck during colonization by a small number of colonists and subsequent inbreeding (Hartl and Clark, 1997). The impact of reproductive systems on genetic diversity in plant populations can also be large. Outcrossing species usually have a high within-population diversity and low population differentiation, whereas selfing species often have low within population diversity and high differentiation among populations (Loveless and Hamrick, 1984; Hamrick and Godt, 1996, 1997; Booy et al., 2000; Till-Bottraud and Gaudeul, 2002; Nybom, 2004).

Molecular studies can also provide valuable information on past biogeographical processes, especially when macrofossils of herbaceous alpine plants are scarce or completely absent (Schönswetter et al., 2005, and references therein). Numerous molecular phylogeographic studies with a focus on potential glacial refugia for Alpine plants in the European Alps have been published over the last years (e.g. Stehlik, 2003; Tribisch and Schönswetter, 2003; Hewitt, 2004), and a recent synthesis demonstrated that glacial refugia were located along the rim of the European Alps as well as in some unglaciated central Alpine areas (Schönswetter et al., 2005).

Molecular data showing genetic subdivisions in species’ ranges might thus provide signals of past biogeographical processes, such as postglacial migration (Comes and...

To explore the effect of natural isolation of alpine habitats on gene flow within and among alpine plant populations, the genetic variability and geographical structure of the Alpine plant species *Campanula thyrsoides* were studied. *Campanula thyrsoides* was selected as the study species because this rare plant species only grows on carbonate-bearing soils in the Alps and adjacent mountain ranges in habitats usually isolated by large distances from each other (Aeschimann et al., 2005; Kuss et al., 2007). Furthermore, seed dispersal capacity of this plant is limited because its seeds lack morphological adaptations for dispersal (Tackenberg and Stocklin 2008). Seeds of *C. thyrsoides* are only dispersed when strong winds, rain or animals shake the seeds out of the cup-like flowering bracts because of the basal capsule-opening mechanism (Kuss et al., 2007). Consequently, gene flow among populations was expected to be low and genetic differentiation to be high. The accompanying study on the breeding system of *C. thyrsoides* showed that the species is predominantly outcrossing (Ægisdóttir et al., 2007a). Another important characteristic of *C. thyrsoides* is that it is monocarpic. Monocarpy reduces flowering population density and consequently limits mating possibilities. The shorter generation time of monocarpic species in combination with a lower population density could lead to a reduction in the effective population size, which should promote population differentiation (Loveless and Hamrick, 1984; Vitalis et al., 2004). In the present study, a large geographical and altitudinal range was covered, and a combination of microsatellite markers and a field-based estimator of pollen dispersal distances was used. Furthermore, tests were carried out for signs of recent bottlenecks and a model-based clustering method implemented in *STRUCTURE* 1.0 (Pritchard et al., 2000) was used to determine the optimal number of genetic clusters present in a population.

Previously, the same leaf material system had been screened using randomly amplified polymorphic DNA (RAPD) markers and a moderately high within-population diversity that was not influenced by population size and altitude was found. Population relatedness showed a clear geographic structuring between populations in the western part of Switzerland and the central and eastern part of Switzerland (Kuss et al., 2008a). In contrast to RAPD markers, which are dominant, microsatellites are co-dominant and this enables a straightforward calculation of allele frequencies, Hardy–Weinberg proportion and the inbreeding coefficient \( F_{IS} \). Another advantage to the use of microsatellites is their selectively neutral and highly polymorphic nature, which, together with co-dominance, makes them very suitable for studying gene flow and population structure (Ouborg et al., 1999; Scribner and Pearce, 2000; Lowe et al., 2004).

The following questions were addressed. (a) How much genetic diversity exists within populations of *C. thyrsoides* and how does it relate to population size and altitude? (b) Does the inbreeding coefficient \( F_{IS} \) indicate inbreeding within the populations? (c) Has the strong isolation of habitats restricted gene flow and consequently led to a strong genetic differentiation among *C. thyrsoides* populations? (d) Is there a geographic subdivision in population differentiation that may be related to postglacial recolonization history?

**MATERIALS AND METHODS**

**Study species**

*Campanula thyrsoides* is a subalpine to alpine plant species, found on carbonate-bearing soils at altitudes between 1000 and 2900 m a.s.l. throughout the European Alps and adjacent mountain ranges (Lauber and Wagner, 2001; Aeschimann et al., 2005). *Campanula thyrsoides* is a diploid \( 2n = 34; \) Rosen, 1931; Larsen, 1954; Gadella, 1964), rosette-forming monocarpic plant species (Kuss et al., 2007). In the year of flowering (mean 7–5 years; range 3–16 years), a 10- to 40-cm-tall inflorescence is formed that carries about 50–200 flowers in a compact spike (Kuss et al., 2007). *Campanula thyrsoides* is exclusively insect pollinated, mainly by bumblebees. It is an outcrossing species, which has protandrous flowers and a gametophytic self-incompatible system, i.e. it is mostly unable to self but can mate with its half-siblings (Ægisdóttir et al., 2007a). It has a limited seed dispersal capacity (Kuss et al., 2007) and the seed dispersal spectra, obtained from simulations with the software *PAPPUS* (Tackenberg and Stocklin, 2008) showed that most seeds (99.99 %) are dispersed within 10 m of the mother plant and only about 15 seeds appear to be dispersed over 1 km (given an average population of 100 individuals and an average fecundity of 15 000 seeds per individual; Kuss et al., 2007).

*Campanula thyrsoides* is considered rare throughout its native range but locally abundant with occasional population sizes exceeding 50 000 individuals. It is predominantly found in pastures and hay-meadows and shows the tendency to ruderalize along road shoulders. In Switzerland it is common in the northern calcareous Alps, whilst in the central siliceous Alps, it is only found on isolated carbonate-bearing outcrops (Kuss et al., 2007). The species is red-listed in different Alpine countries. In Germany, *C. thyrsoides* is protected at the national, in Austria and Switzerland at regional level (Kuss et al., 2007).

**Sampling sites**

Leaf material was sampled from 32 populations in Switzerland ranging from the Jura Mountains in the west to the easternmost part of the Swiss Alps (Table 1 and Fig. 1). In each population, a constant number of individual plants \( n = 23 \) was randomly sampled. The altitude of the sampled populations ranged from 1340 to 2430 m a.s.l. The leaf material was dried in silica gel and stored at room temperature. Population size was assessed either by complete census or through extrapolation of average density counts from population subsets (Table 1).

**DNA extraction and PCR amplification**

DNA was extracted from 10 mg of silica-gel dried and milled (Retsch MM300) leaf tissue using a DNaseasy Plant 96 Kit (Qiagen, Hombrechtikon, Switzerland). To remove polyphenols, which may adversely affect PCR amplification, the
first extraction step of the manufacturer’s protocol was modified by adding 25 mg of polyvinylpyrrolidone (Fluka, Buchs, Switzerland) to the 600 μL of lysis buffer of each sample. The DNA was quantified using a NanoDrop ND-1000 Spectrophotometer. Samples were screened using seven polymorphic microsatellite loci of which five were used for the final analyses: Camphy1, Camphy3, Camphy5, Camphy13 and Camphy15 (Ægisdóttir et al., 2007b). That was done in order to minimize error rate and avoid pseudoreplication in the event of linkage disequilibrium [as recommended by Bonin et al. (2004) and Selkoe and Toonen (2006)].

Polymerase chain reaction (PCR) amplification was performed in a 10-μL reaction volume containing 15 ng of genomic DNA, 0.125 μM each of forward and reverse primers, 1 μL of 10× PCR buffer, 150 μM dNTP and 0.5 U HotsTarTaq (Qiagen, Hombrechtikon, Switzerland). After a denaturation step at 95°C for 15 min, PCR was performed for 30 cycles: 30 s annealing at a locus-specific temperature (56°C for Camphy1, 3, 5, 6, 9 and 12; 60°C for Camphy13 and Camphy15), 30 s at 72°C, and 30 s at 95°C. The PCR ended with 1 min at a locus-specific temperature, followed by a final 30-min extension step at 72°C. Horizontal gel electrophoresis of PCR products was performed using Spreadex™ gels with a resolution of 2 bp in a SEA-2000™ submerged gel electrophoresis system (Elchrom Scientific AG, Cham, Switzerland). Ethidium bromide (1 μg/mL)-stained banding patterns were observed under UV light and analysed by careful manual verification of each gel at least twice.

All ambiguous genotyping results were repeated to minimize genotyping errors, and low quality or unreliable DNA samples and markers discarded. The genotyping error rate was calculated by re-extracting and re-amplifying 39 randomly chosen plants (5.3% of the dataset). Subsequently allelic differences were calculated between those 39 plants and the original datasets. The error rate was estimated to be 6.1% and was due to genotyping and scoring errors. Eventually, some individuals had to be excluded from the original dataset. The excluded individuals had microsatellite profiles with only one scored allele at a specific locus. Such loci cannot be included in analyses that require information of two alleles per locus (such as Hs, Hardy–Weinberg equilibrium, and calculation in the program STRUCTURE). The reason for excluding such individuals was due to stuttering or smearing of bands (e.g. the ‘second’ allele could not be

<table>
<thead>
<tr>
<th>Location*</th>
<th>Population code</th>
<th>Co-ordinates1</th>
<th>Altitude (m a.s.l.)</th>
<th>Population size</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Col du Marchairux, VD</td>
<td>JUM</td>
<td>508’900/156’400</td>
<td>1440</td>
<td>1000</td>
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<tr>
<td>(2) Les Amburnez, VD</td>
<td>JUA</td>
<td>507’480/155’100</td>
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<td>1000</td>
</tr>
<tr>
<td>(3) Pré du Rolle, VD</td>
<td>JUR</td>
<td>508’983/155’652</td>
<td>1377</td>
<td>150</td>
</tr>
<tr>
<td>(4) Pres de Four, VD</td>
<td>JUF</td>
<td>494’800/148’450</td>
<td>1430</td>
<td>10000</td>
</tr>
<tr>
<td>(5) Col du Janton, VD</td>
<td>JAA</td>
<td>564’830/145’050</td>
<td>1630</td>
<td>100</td>
</tr>
<tr>
<td>(6) Col du Janton, VD</td>
<td>JAC</td>
<td>564’589/144’944</td>
<td>1670</td>
<td>80</td>
</tr>
<tr>
<td>(7) Lac du Fully, VS</td>
<td>FUL</td>
<td>574’000/113’200</td>
<td>2100</td>
<td>500</td>
</tr>
<tr>
<td>(8) Trient, Les Tsепpes, VS</td>
<td>TRI</td>
<td>564’350/099’500</td>
<td>2020</td>
<td>50</td>
</tr>
<tr>
<td>(9) Lac du Moiry, VS</td>
<td>MOI</td>
<td>609’932/109’638</td>
<td>2266</td>
<td>50000</td>
</tr>
<tr>
<td>(10) Stockhorn, BE</td>
<td>STO</td>
<td>607’737/171’103</td>
<td>1980</td>
<td>100</td>
</tr>
<tr>
<td>(11) Schynige Platte, BE</td>
<td>SPO</td>
<td>636’225/167’625</td>
<td>1990</td>
<td>600</td>
</tr>
<tr>
<td>(12) Schynige Platte, BE</td>
<td>SPU</td>
<td>636’600/167’150</td>
<td>1890</td>
<td>500</td>
</tr>
<tr>
<td>(13) Furka, UR/VS</td>
<td>FUR</td>
<td>674’850/158’825</td>
<td>2430</td>
<td>30000</td>
</tr>
<tr>
<td>(14) Unterschächen, Bütztlichöpf, UR</td>
<td>UNB</td>
<td>702’500/193’200</td>
<td>1900</td>
<td>500</td>
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<tr>
<td>(15) Langwies, Listboden, GR</td>
<td>LAL</td>
<td>776’750/191’510</td>
<td>2000</td>
<td>300</td>
</tr>
<tr>
<td>(16) Langwies, Strassberg, GR</td>
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<td>775’875/190’550</td>
<td>1870</td>
<td>7000</td>
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<tr>
<td>(17) Langwies, Holzbiel, GR</td>
<td>LAH</td>
<td>779’010/188’875</td>
<td>1700</td>
<td>50</td>
</tr>
<tr>
<td>(18) Vals, Pels, GR</td>
<td>VAL</td>
<td>735’375/160’425</td>
<td>1850</td>
<td>100</td>
</tr>
<tr>
<td>(19) Safiental, GR</td>
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<td>742’851/174’289</td>
<td>1857</td>
<td>50</td>
</tr>
<tr>
<td>(20) Medels, Parjurs, GR</td>
<td>MED</td>
<td>742’800/157’700</td>
<td>1870</td>
<td>50</td>
</tr>
<tr>
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<td>MOM</td>
<td>779’668/173’708</td>
<td>1961</td>
<td>45</td>
</tr>
<tr>
<td>(22) Monstein, Fanexmeder, GR</td>
<td>MOF</td>
<td>780’750/174’910</td>
<td>2220</td>
<td>250</td>
</tr>
<tr>
<td>(23) Parsennmeder, GR</td>
<td>PMA</td>
<td>784’030/191’473</td>
<td>1995</td>
<td>5000</td>
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<tr>
<td>(24) Parsennmeder, GR</td>
<td>PMB</td>
<td>784’478/191’548</td>
<td>1910</td>
<td>100</td>
</tr>
<tr>
<td>(25) Churwalden, Joch, GR</td>
<td>CHJ</td>
<td>762’300/185’100</td>
<td>1890</td>
<td>150</td>
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<tr>
<td>(26) St. Antonien, GR</td>
<td>STA</td>
<td>782’203/201’989</td>
<td>1943</td>
<td>250</td>
</tr>
<tr>
<td>(27) Alp Laret, GR</td>
<td>LAR</td>
<td>784’234/153’944</td>
<td>2180</td>
<td>300</td>
</tr>
<tr>
<td>(28) Albula Pass, Naz, GR</td>
<td>NAZ</td>
<td>778’193/162’751</td>
<td>1755</td>
<td>150</td>
</tr>
<tr>
<td>(29) School, La Motta, GR</td>
<td>SCM</td>
<td>816’400/188’400</td>
<td>2142</td>
<td>2000</td>
</tr>
<tr>
<td>(30) Pian, Prui, GR</td>
<td>FTA</td>
<td>812’505/187’750</td>
<td>2100</td>
<td>150</td>
</tr>
<tr>
<td>(31) Tschlin, Alp Tea, GR</td>
<td>TEA</td>
<td>828’250/198’250</td>
<td>2200</td>
<td>150</td>
</tr>
<tr>
<td>(32) Tschlin, Alp Tea, GR</td>
<td>TEB</td>
<td>827’800/198’000</td>
<td>2150</td>
<td>200</td>
</tr>
</tbody>
</table>

A constant number of 23 C. thyrsoides individuals was sampled at each location.
* The two-letter code after the localities indicates the canton in Switzerland.
† Co-ordinates according to the Swiss topographical maps (Bundesamt für Landestopographie, Wabern, Switzerland).

TABLE 1. Geographical location, population code, co-ordinates, altitude and estimated population size of 32 populations of Campanula thyrsoides in Switzerland
scored correctly) or due to evidence of null alleles (i.e. false positive homozygous genotypes). The total percentage of excluded data for each locus was as follows: 1, 3.9% of all individuals; 3, 0.9%; 5, 4%; 6, 4.8%; 15, 10.5%; 13, 7.5%. Nevertheless, individual profiles with only one allele can still be incorporated in population genetic programs, e.g. for estimation of population differentiation ($F_{ST}$, $G_{ST}$ and $G_{ST}^2$; see below).
Analysis of overall genetic variation

Molecular diversity was calculated within populations with (a) mean number of alleles per locus \(N_a\), (b) \(H_E\) (observed heterozygosity), (c) \(H_E^*\) (expected heterozygosity under Hardy–Weinberg equilibrium) and (d) inbreeding coefficient \(F_{IS}\). Moreover, genetic diversity was calculated among populations with (e) \(F_{ST}\) using the method of Weir and Cockerman (1984) and (f) Nei’s \(G_{ST}\) (Nei, 1973). Both \(F_{ST}\) and \(G_{ST}\) were calculated in genetix version 4.05 (Belkhir et al., 1996–2002). Since the standardized measurement of \(G_{ST}\) allows more appropriate measures of genetic differentiation than \(G_{ST}\) or \(F_{ST}\) (Hedrick, 2005; Heller and Siegismund, 2009), \(G_{ST}\) was calculated (see eqn 4b in Hedrick, 2005). Confidence intervals (95%) were calculated by bootstrapping 1000 times over all loci (for \(F_{IS}\) within each population, for \(F_{ST}\) within each population and for \(F_{ST}\) globally; Weir and Cockerman, 1984; Belkhir et al., 1996–2002).

Using GENEPOL on the web, version 3.4 (Raymond and Rousset, 1995), the probability for linkage disequilibrium was assessed using 5000 dememorizations, 1000 batches and 5000 iterations per batch, and the number of alleles per locus and and deviations from Hardy–Weinberg expectations for each pair of loci calculated across all populations using a Fisher exact test. Confidence intervals were calculated with the Markov chain method (Guo and Thompson, 1992). Bonferroni correction for multiple comparisons was applied in both cases to a significance level of \(P < 0.05\).

Moreover, the effect of population size and altitude on molecular variation (\(H_E\), \(F_{IS}\) and \(N_a\)) was assessed by performing single linear regression using the software R 2.0.0 (R Development Core Team, 2004).

For a species that lives in spatially isolated and sometimes small populations within the alpine landscape (like C. thyrsoides), the number of plants colonizing isolated habitats might be limited, which could cause founder effect. Bottlenecks were searched within the populations studied by applying the mode-shift test implemented in the software BOTTLENECK, version 1.2.02 (Piry et al., 1999).

Geographical structure

To explore whether subsets of populations were confined to discrete geographical regions (two or three gene pools) as found when using the RAPD markers, a model-based clustering method implemented in STRUCTURE 1.0 was used (Pritchard et al., 2000). To determine the optimal number of genetic clusters present in a population, STRUCTURE divides the sampled individuals into a number of clusters \(K\) based only on multilocus genotypic data. Twenty independent runs were conducted for each \(K\) between one and five, and the results from each run plotted with the highest likelihood value (see the Appendix). The admixture model was used and allele frequencies, a burn-in period and Markov chain Monte Carlo (MCMC) iterations of 10 000 each were correlated. The ad hoc statistic \(\Delta K\) was used to identify the most likely number of clusters in the dataset (Evanno et al., 2005).

Pairwise \(F_{ST}\) was calculated and differentiation tested between all pairs of populations with a permutation test included in ARLEQUIN 3.1 (Excoffier et al., 2005). To test for isolation by distance among populations, the independence between pairwise \(F_{ST}\) and geographical distances was examined by running 10 000 permutations using a modified Mantel’s test (Mantel, 1967) included in the program ISOLDE in GENEPOL (version 3.1c) (Raymond and Rousset, 1995).

Furthermore, tests were carried out for genetic differentiation among populations and between regions (east and west), as observed using STRUCTURE 1.0 and with analysis of molecular variance (AMOVA) (Excoffier et al., 1992), as applied in ARLEQUIN 3.1, using 1000 permutations of the individual (Schneider et al., 2000).

Data on dispersal

Pollen dispersal distance was measured in the field during 1 d in the summer of 2004 and 2 d in the summer of 2005 using fluorescent powder of similar particle size as pollen (Stockhouse, 1976; fluorescent dye from Radiant Colour, Houthalen, Belgium). The study took place on the Furka Pass in the Central Swiss Alps, which holds a big C. thyrsoides population extending over a large area. In both summers, clear sunny days in the middle of the flowering period of C. thyrsoides were chosen for the study. On 6 July 2004, six plants were selected to serve as donors. In 2005, the study took place on 15 July (one donor plant), and 17 July (two donor plants). On each donor plant, the pollen of every open flower (mean number of flowers per plant = 28.33, s.d. = 11.57) was painted with fluorescent powder early in the morning. The population was divided into four parts of equal area and one or two plants were randomly selected within each part of the population. If there were two plants selected within a single part, they were painted with powder of dissimilar colours (pink, yellow, blue and green). Similar colours were only used on plants which were further away (>150 m) from each other to prevent false results. Throughout the day, pollinators frequented the flowers and dispersed the fluorescent powder. After sunset, traces of the fluorescent powder on C. thyrsoides flowers were identified using UV torches. Then the number of plants and flowers visited per plant was counted and the distances to the initially marked plants measured. This method approximates minimum pollen dispersal via insects. However, it is limited in estimating long-distance dispersal due to logistic constraints. For long-distance seed dispersal by wind, modeling work that incorporated turbulences and topographic effects was used (Kuss et al., 2007; Tackenberg and Stöcklin, 2008).

On the same sunny days as the fluorescent powder experiment was done, the number of insects that frequented the C. thyrsoides flowers were monitored and counted. The observations took place in the middle of the day and the observed insect groups were bumblebees, small Diptera spp., beetles and ants. Eleven 0.5- to 1.0-m² plots were observed during 5.5 h of observation altogether. If flower density was high (>10 flowering C. thyrsoides plants within 1 m²), 0.5-m² plots were used so that it was possible to observe all visits by pollinators. However, when <10 flowering C. thyrsoides plants where found within an area of 1 m², 1-m² plots were used for observations.


Table 2. Mean number of alleles per locus ($N_a$), expected heterozygosity ($H_e$), observed heterozygosity ($H_o$) and inbreeding coefficient ($F_{IS}$) for each studied population of Campanula thyrsoides across five loci.

<table>
<thead>
<tr>
<th>Site</th>
<th>$N_a$</th>
<th>$H_e$</th>
<th>$H_o$</th>
<th>$F_{IS}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7-4</td>
<td>0.756</td>
<td>0.682</td>
<td>0.018***</td>
</tr>
<tr>
<td>2</td>
<td>7-4</td>
<td>0.767</td>
<td>0.763</td>
<td>0.021</td>
</tr>
<tr>
<td>3</td>
<td>7-2</td>
<td>0.750</td>
<td>0.798</td>
<td>0.024</td>
</tr>
<tr>
<td>4</td>
<td>5-6</td>
<td>0.666</td>
<td>0.673</td>
<td>0.022</td>
</tr>
<tr>
<td>5</td>
<td>9-6</td>
<td>0.813</td>
<td>0.712</td>
<td>0.017***</td>
</tr>
<tr>
<td>6</td>
<td>9-6</td>
<td>0.793</td>
<td>0.849</td>
<td>0.024***</td>
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<tr>
<td>7</td>
<td>7-6</td>
<td>0.767</td>
<td>0.821</td>
<td>0.024</td>
</tr>
<tr>
<td>8</td>
<td>7-8</td>
<td>0.741</td>
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<tr>
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<td>8-8</td>
<td>0.832</td>
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<tr>
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<tr>
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<td>7-5</td>
<td>0.769</td>
<td>0.809</td>
<td>0.025</td>
</tr>
<tr>
<td>21</td>
<td>6-0</td>
<td>0.701</td>
<td>0.671</td>
<td>0.020</td>
</tr>
<tr>
<td>22</td>
<td>6-6</td>
<td>0.740</td>
<td>0.791</td>
<td>0.023</td>
</tr>
<tr>
<td>23</td>
<td>9-2</td>
<td>0.786</td>
<td>0.734</td>
<td>0.020**</td>
</tr>
<tr>
<td>24</td>
<td>9-2</td>
<td>0.780</td>
<td>0.806</td>
<td>0.023</td>
</tr>
<tr>
<td>25</td>
<td>9-0</td>
<td>0.787</td>
<td>0.762</td>
<td>0.021</td>
</tr>
<tr>
<td>26</td>
<td>7-4</td>
<td>0.739</td>
<td>0.645</td>
<td>0.017**</td>
</tr>
<tr>
<td>27</td>
<td>8-0</td>
<td>0.749</td>
<td>0.722</td>
<td>0.020</td>
</tr>
<tr>
<td>28</td>
<td>6-4</td>
<td>0.718</td>
<td>0.785</td>
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</tr>
<tr>
<td>29</td>
<td>6-8</td>
<td>0.750</td>
<td>0.761</td>
<td>0.022***</td>
</tr>
<tr>
<td>30</td>
<td>7-0</td>
<td>0.715</td>
<td>0.771</td>
<td>0.024</td>
</tr>
<tr>
<td>31</td>
<td>7-2</td>
<td>0.761</td>
<td>0.771</td>
<td>0.022</td>
</tr>
<tr>
<td>32</td>
<td>7-2</td>
<td>0.746</td>
<td>0.734</td>
<td>0.021***</td>
</tr>
<tr>
<td>Mean</td>
<td>8-0</td>
<td>0.762</td>
<td>0.763</td>
<td>0.022</td>
</tr>
</tbody>
</table>

$^1$ Statistically significant deviations from Hardy–Weinberg expectations by $P < 0.01$ (**) and $P < 0.001$ (**).

RESULTS

Overall microsatellite diversity

Five loci were genotyped in each of 736 individuals of *C. thyrsoides* and 100 alleles in total and 15–28 alleles per locus with an average of 20 alleles detected. The observed heterozygosity ($H_o$) over all populations was high (mean $H_o$ 0.763; range 0.645–0.849) and deviation from expected heterozygosity ($H_e$) was small (mean $H_e$ 0.762; range 0.666–0.847) (Table 2). There was no significant difference in expected heterozygosity ($H_e$) among populations ($F = 1.72$; $P = 0.20$).

The average inbreeding coefficient was relatively low ($F_{IS} = 0.022$), ranging from 0.017 to 0.025 at the population level (Table 2). Eleven out of 32 populations deviated significantly from Hardy–Weinberg equilibrium (Table 2). None of the studied populations showed significant signs of a recent bottleneck. No evidence was found for linkage disequilibrium between the studied loci.

Results from linear regression indicated that altitude (m a.s.l.) had no influence on molecular variation within populations ($H_e$, $r = 0.2$, $P = 0.29$; $F_{IS}$, $r = 0.14$, $P = 0.89$; $N_a$, $r = 0.06$, $P = 0.72$). Similarly, population size had no influence on the molecular variation ($H_e$, $r = 0.1$, $P = 0.078$; $F_{IS}$, $r = 0.17$, $P = 0.37$; $N_a$, $r = 0.105$, $P = 0.56$).

Genetic differentiation and geographic structure

Geographic structure based on assignment tests in STRUCTURE revealed that two genetic clusters ($K = 2$) have the best ad hoc statistical fit ($\Delta K$; Evanno et al., 2005). Here, a clear west–east division was found with populations numbered 1–9 clustering together in western Switzerland and populations numbered 10–32 clustering in central and eastern Switzerland (Fig. 1, top). For $K = 3$ and $K = 4$, the west–east divisions were still very clear but other divisions with new gene pools appeared in the east (one regional gene pool for $K = 3$, and two for $K = 4$; see Fig. 1, bottom, for $K = 3$).

The average genetic differentiation in the populations studied was comparably low, based either on $F_{ST} = 0.10$ or $G_{ST} = 0.12$ algorithms, though considerably higher in the case of the standardized $G_{ST} = 0.53$.

Genetic differentiation between the populations correlated significantly with geographical distances (Mantel test based on pairwise $F_{ST}$; $r = 0.62$; $P < 0.001$; Fig. 2A). When the two regions (east and west) were tested separately, there was still a significant correlation between the geographical distances and the pairwise $F_{ST}$ in the eastern region ($r = 0.26$; $P < 0.05$; Fig. 2B). In the western region, however, the relationship was only marginally significant ($r = 0.26$; $P = 0.052$; Fig. 2C). AMOVA results are listed in Table 3.

Dispersal

In total, 1102 dye transfers among flowers were recorded on a total of 173 different plants. On average, the powder was found on 6-4 flowers per plant (s.e. = 0.48). The frequency of observed pollen dispersal distances was highest within the first 2 m from the marked plant but decreased markedly with increasing distance. The average dispersal distance was 4.85 m (s.e. = 0.54), the shortest 0.13 m, and the longest observed dispersal distance 39 m (Fig. 3). Bumblebees, which were observed collecting a large amount of pollen from flowers, were the prime flower visitor, accounting for 60% of the total visits, followed by small Diptera species whose contribution measured about 30% of the total visits. Other insect groups accounted for a smaller number of visits and are unlikely to be pollinators (beetles, 7%; ants, 3%).

DISCUSSION

Genetic diversity within populations

Higher levels of genetic diversity were found within populations of *C. thyrsoides* (expected heterozygosity; $H_e = 0.762$) than many other microsatellite-based plant studies (e.g. Sun and Salomon, 2003; Mengoni et al., 2000; Galeuchet et al., 2005; Gao, 2005). A review by Nybom (2004) reported, for microsatellite studies, an average $H_e$ of 0.55 for short-lived perennials and $H_e$ of 0.65 for outcrossing plant species. No indication of genetic erosion or recent
bottlenecks were found despite the species rareness and isolation of some of its populations.

Population size did not affect molecular diversity in *C. thyrsoides* despite the theoretical prediction that small populations might lose genetic variation due to genetic drift, founder effects and population bottlenecks (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Young et al., 1996; Lowe et al., 2004). Although drift could be expected in some of the *C. thyrsoides* populations studied here because of their small size (see Table 1), no significant signs of bottlenecks or founder effects were found. Other studies on Alpine plant species that include comparisons of genetic diversity and population sizes of similar size range have either detected (*Lloydia serotina*, Jones and Gliddon, 1999; *Eryngium alpinum*, Gaudeul et al., 2000; *Trollius europaeus*, Despres et al., 2002; *Sesleria albicans*, Reisch et al., 2002) or have not detected any correlation (*Geum reptans*, Pluess and Stöcklin, 2004; *Epilobium fleischeri*, Kuss et al., 2008a). This inconsistent pattern among alpine plants may be a result of different sampling parameters such as sample size and marker system and/or biological traits such as breeding system and life span (Nybom, 2004). In *C. thyrsoides*, the longevity of individuals despite its monocarp, the desynchronized flowering of cohorts (generation overlap) are likely factors that prevent negative effects of small size or isolation of populations on genetic diversity (Kuss et al., 2008b).

The high within-population diversity in *C. thyrsoides* is also likely to be influenced by some life-history traits of the species, especially the type of breeding system, which has been shown to strongly affect the distribution and magnitude of genetic diversity in plant populations. Outcrossing plant species tend to have higher genetic variation within populations, whereas populations of selfing species or species with a mixed mating system are often genetically less variable (Loveless and Hamrick, 1984; Hamrick and Godt, 1996, 1997; Booy et al., 2000; Till-Bottraud and Gaudeul, 2002; Nybom, 2004; Duminil et al., 2007). Since *C. thyrsoides* is predominantly outbreeding (*Ægisdóttir et al., 2007a*) with low inbreeding coefficient (*FIS* = 0.022), we suggest that the breeding system of *C. thyrsoides* plays an important role in driving the high within-population diversity. Furthermore, the high number of traces of fluorescent powder observed in the field (1102 times on 173 plants) with occasional flight distances of 39 m indicate that pollinators (especially bumblebees) contribute to the high genetic variation within populations.

**Heterozygote deficiency**

Whilst the inbreeding coefficient (*FIS*) was low in all populations (average *FIS* = 0.022), most values were positive and 11 populations out of 32 significantly deviated from Hardy–Weinberg equilibrium. Heterozygote deficiency in a population can occur as a result of inbreeding, selection against heterozygotes, the Wahlund effect (reduction of heterozygosity in a population caused by subpopulation structure) or the presence of null alleles (Sun and Salomon, 2003, and references therein). In our opinion, the most plausible explanation for the observed deviation from Hardy–Weinberg equilibrium in populations of *C. thyrsoides* is the ability of this species to mate with half-sibs (*Ægisdóttir et al., 2007a*). This allows some inbreeding which leads to more homozygotes than expected. Yet, other explanations (like the presence of null alleles) may also contribute to the lack of heterozygosity (see Materials and methods).

**Genetic differentiation in a landscape context**

The genetic differentiation values of *C. thyrsoides* in this study showed rather low values of 0.10 for *FST* and 0.12 for *GST*. It has, however, been argued that the high levels of within-population heterozygosity usually found with microsatellites yield low differentiation indices among populations.
The clear geographical boundary existing between populations in western Switzerland and populations in central/eastern Switzerland (K = 2; Fig. 1A), had already been found with RAPD profiles (Kuss et al., 2008a), and remained after increasing the number of groups in STRUCTURE to K = 3 (Fig. 1B) and K = 4 (figure not shown). AMOVA indicated that 5.4% of the genetic diversity was found among regions. There were no indications that the two groups of populations relate to a particular structure in the landscape. Since the observed west–east division in the populations also did not correspond to the geographical division between the Jura mountains in the west and the Alps in the east, we consider it most likely that this genetic boundary arose as a result of postglacial colonization history, as it corresponds to the glacial refugia map for mountain plants in the European Alps proposed by Schönswetter et al. (2005).

Using STRUCTURE, another but less-pronounced gene pool was identified in the eastern-most populations (Fig. 1B) using K = 3. Using four gene pools (K = 4), moreover, one more gene pool was identified that was regional in the eastern part of Switzerland (figure not shown). It remains to be explored to what extent the emerging easternmost Swiss gene pools correspond with the split between the eastern and central Alps as outlined by Schönswetter et al. (2005).

Because of the poor dispersal capacity of C. thyrsoides, the isolation of its suitable habitats in the Alpine landscape and the considerable genetic differentiation found in its populations, we find it most likely that the genetic pattern found in C. thyrsoides is at least partly reflecting a historical gene flow over a timescale of several hundred or thousands of years. Possibly, this gene flow has occurred since the end of the last glacial period (10 000 years ago) when the species invaded Switzerland from at least two different glacial refugia as indicated by the geographical pattern observed with STRUCTURE (Fig. 1).

Conclusions

A high within-population genetic diversity is reported for the rare monocarpic perennial Campanula thyrsoides in the Swiss Alps, which can best be explained by long-lived individuals, overlapping generations and the outbreeding behaviour of the species. Despite living in isolated populations of different sizes and at contrasting altitudes in the Alpine landscape, neither isolation of habitats nor population size or altitude affected genetic variability within the studied populations.

The considerable genetic differentiation and the isolation by distance relationship observed in the present study show that gene flow is restricted among the C. thyrsoides populations. This was to be expected in a monocarpic perennial that lives in isolated populations in the Alpine landscape and has poor seed dispersal capacities. In addition, a clear geographical boundary was found among populations in western versus central/eastern regions of Switzerland, which corresponds well with the postglacial colonization history of many other Alpine plants (Schönswetter et al., 2005).

The present molecular study found no indication of genetic depletion, even within small populations of C. thyrsoides, despite the rareness of this species in the alpine landscape. Nevertheless, the considerable genetic differentiation among populations and the clear west–east differentiation between two population groups should be considered in future conservation efforts for this species.

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APPENDIX

Mean values of 20 runs (± s.d.) of ln probability of data [ln P(D)] for K from 1 to 5 as implemented in STRUCTURE on Campanula thyrsoides.

Mean values of 20 runs (± s.d.) of ln probability of data [ln P(D)] for K from 1 to 5 as implemented in STRUCTURE on Campanula thyrsoides.