

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

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- **Background and Aims** Gene flow and genetic variability within and among alpine plant populations can be greatly influenced by the steep environmental gradients and heterogeneous topography of alpine landscapes. In this study, the effects are examined of natural isolation of alpine habitats on genetic diversity and geographic structure in populations of *C. thyrsoides*, a rare and isolated European Alpine monocarpic perennial with limited seed dispersal capacity.
- **Methods** Molecular diversity was analysed for 736 individuals from 32 populations in the Swiss Alps and adjacent Jura mountains using five polymorphic microsatellite loci. Pollen flow was estimated using pollen grain-sized fluorescent powder. In addition, individual-based Bayesian approaches were applied to examine population structure.
- **Key Results** High within-population genetic diversity ( $H_E = 0.76$ ) and a relatively low inbreeding coefficient ( $F_{IS} = 0.022$ ) were found. Genetic differentiation among populations measured with a standardized measure was considerable ( $G'_{ST} = 0.53$ ). A significant isolation-by-distance relationship was found ( $r = 0.62$ ,  $P < 0.001$ ) and a significant geographic sub-structure, coinciding with proposed postglacial migration patterns. Altitudinal location and size of populations did not influence molecular variation. Direct measures of pollen flow revealed that insect-mediated pollen dispersal was restricted to short distances within a population.
- **Conclusions** The natural isolation of suitable habitats for *C. thyrsoides* restricts gene flow among the populations as expected for a monocarpic species with very limited seed dispersal capacities. The observed high within-population genetic diversity in this rare monocarpic perennial is best explained by its outcrossing behaviour, long-lived individuals and overlapping generations. Despite the high within-population genetic diversity, the considerable genetic differentiation and the clear western–eastern differentiation in this species merits consideration in future conservation efforts.

**Key words:** Alpine plant, *Campanula thyrsoides*, genetic diversity, gene flow, genetic differentiation, glacial history,  $G'_{ST}$ , habitat isolation, microsatellites, monocarpy, SSR.

### 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; Tribsch 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

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Kadereit, 1998; Stehlik, 2003; Tribsch and Schönswetter, 2003).

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 thyrsooides* were studied. *Campanula thyrsooides* 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 Stöcklin 2008). Seeds of *C. thyrsooides* 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. thyrsooides* showed that the species is predominantly outcrossing (Ægisdóttir *et al.*, 2007a). Another important characteristic of *C. thyrsooides* 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. thyrsooides* 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. thyrsooides* populations? (d) Is there a geographic subdivision in population

differentiation that may be related to postglacial recolonization history?

## MATERIALS AND METHODS

### Study species

*Campanula thyrsooides* 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 thyrsooides* 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 thyrsooides* 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 Stöcklin, 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 thyrsooides* 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. thyrsooides* 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 DNeasy Plant 96 Kit (Qiagen, Hombrechtikon, Switzerland). To remove polyphenols, which may adversely affect PCR amplification, the

TABLE 1. Geographical location, population code, co-ordinates, altitude and estimated population size of 32 populations of *Campanula thyrsoidea* in Switzerland

Location*	Population code	Co-ordinates†	Altitude (m a.s.l.)	Population size
(1) Col du Marchairux, VD	JUM	508' 900/156' 400	1440	1000
(2) Les Amburnez, VD	JUA	507' 480/155' 100	1340	1000
(3) Pre du Rolle, VD	JUR	508' 983/155' 652	1377	150
(4) Pres de Four, VD	JUF	498' 400/148' 450	1430	10 000
(5) Col du Jamon, VD	JAA	564' 830/145' 050	1630	100
(6) Col du Jamon, VD	JAC	564' 589/144' 944	1670	80
(7) Lac du Fully, VS	FUL	574' 000/113' 200	2100	500
(8) Trient, Les Tseppes, VS	TRI	564' 350/099' 500	2020	50
(9) Lac du Moiry, VS	MOI	609' 932/109' 638	2266	50 000
(10) Stockhorn, BE	STO	607' 737/171' 103	1980	100
(11) Schynige Platte, BE	SPO	636' 225/167' 625	1990	600
(12) Schynige Platte, BE	SPU	636' 600/167' 150	1890	500
(13) Furka, UR/VS	FUR	674' 850/158' 825	2430	30 000
(14) Unterschächen, Butzlihöpf, UR	UNB	702' 500/193' 200	1900	500
(15) Langwies, Listboden, GR	LAL	776' 750/191' 510	2000	300
(16) Langwies, Strassberg, GR	LAS	775' 875/190' 550	1870	7000
(17) Langwies, Holzbüel, GR	LAH	775' 010/188' 875	1700	50
(18) Vals, Peil, GR	VAL	735' 375/160' 425	1850	100
(19) Safiental, GR	SAF	742' 851/174' 289	1857	50
(20) Medels, Parjurs, GR	MED	742' 800/157' 700	1870	50
(21) Monstein, Mäschenboden, GR	MOM	779' 668/173' 708	1961	45
(22) Monstein, Fanexmeder, GR	MOF	780' 750/174' 910	2220	250
(23) Parsennmeder, GR	PMA	784' 030/191' 473	1995	5000
(24) Parsennmeder, GR	PMB	784' 478/191' 548	1910	100
(25) Churwalden, Joch, GR	CHJ	762' 300/185' 100	1890	150
(26) St. Antönien, GR	STA	782' 203/201' 989	1943	250
(27) Alp Laret, GR	LAR	784' 234/153' 944	2180	300
(28) Albula Pass, Naz, GR	NAZ	778' 193/162' 751	1755	150
(29) Schuol, La Motta, GR	SCM	816' 400/188' 400	2142	2000
(30) Ftan, Prui, GR	FTA	812' 505/187' 750	2100	150
(31) Tschlin, Alp Tea, GR	TEA	828' 250/198' 250	2200	150
(32) Tschlin, Alp Tea, GR	TEB	827' 800/198' 000	2150	200

A constant number of 23 *C. thyrsoidea* 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).

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 (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer. Samples were screened using seven polymorphic microsatellite loci of which five were used for the final analyses: Camphy 1, Camphy 3, Camphy 5, Camphy 13 and Camphy 15 (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 Camphy 1, 3, 5, 6, 9 and 12; 60 °C for Camphy 13 and 15), 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  $H_E$ , 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

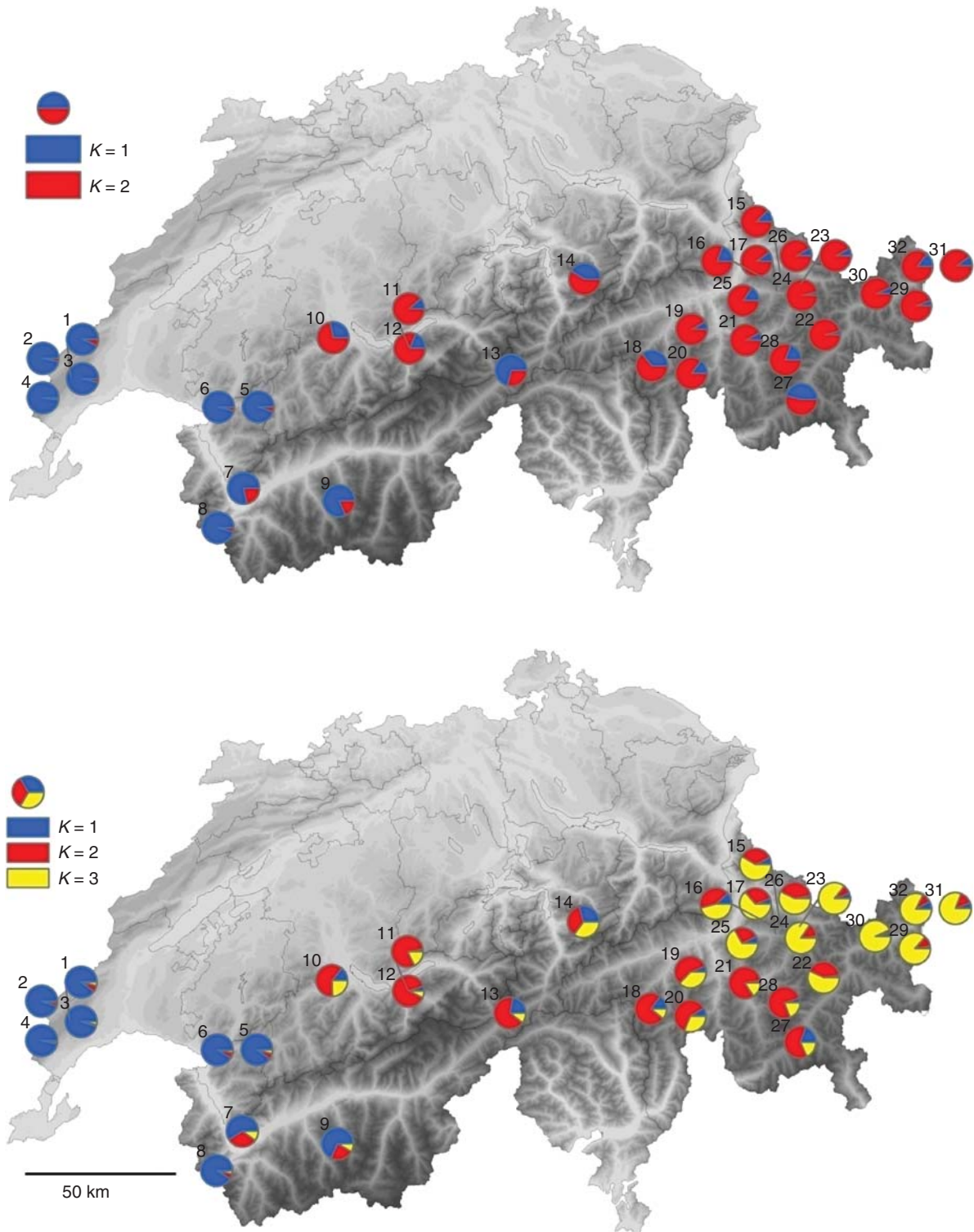


FIG. 1. Geographical structure of 32 populations of *Campanula thyrsooides* in Switzerland based on the clustering method implemented in STRUCTURE 1.0. The figure shows the distribution for  $K = 2$  and  $K = 3$ , where  $K =$  genetic clusters. The numbers indicate the location in Table 1. The lightest grey colour is at 190 m a.s.l. and the altitude increases with darker colour up to about 4800 m a.s.l.

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  $G'_{ST}$ ; see below).

### Analysis of overall genetic variation

Molecular diversity was calculated within populations with (a) mean number of alleles per locus ( $N_a$ ), (b)  $H_O$  (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 Cockerham (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 Siegmund, 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 Cockerham, 1984; Belkhir *et al.*, 1996–2002).

Using GENEPOP 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 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. thyrsooides*), 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 GENEPOP (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. thyrsooides* population extending over a large area. In both summers, clear sunny days in the middle of the flowering period of *C. thyrsooides* 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. thyrsooides* 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, modelling 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. thyrsooides* 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-m<sup>2</sup> plots were observed during 5.5 h of observation altogether. If flower density was high (>10 flowering *C. thyrsooides* plants within 1 m<sup>2</sup>), 0.5-m<sup>2</sup> plots were used so that it was possible to observe all visits by pollinators. However, when <10 flowering *C. thyrsooides* plants were found within an area of 1 m<sup>2</sup>, 1-m<sup>2</sup> 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 thyrsoidea* across five loci

Site	$N_a$	$H_E$	$H_O$	$F_{IS}^\dagger$
1	7.4	0.756	0.682	0.018***
2	7.4	0.767	0.763	0.021
3	7.2	0.750	0.798	0.024
4	5.6	0.666	0.673	0.022
5	9.6	0.813	0.712	0.017***
6	9.8	0.793	0.849	0.024***
7	7.6	0.767	0.821	0.024
8	7.8	0.741	0.773	0.023
9	8.8	0.832	0.817	0.021
10	10.2	0.847	0.841	0.021
11	7.8	0.778	0.690	0.018***
12	7.6	0.729	0.728	0.022***
13	8.4	0.797	0.739	0.019***
14	10.0	0.834	0.813	0.021***
15	9.6	0.766	0.819	0.024
16	9.4	0.798	0.799	0.021
17	9.0	0.745	0.778	0.022
18	8.4	0.766	0.835	0.024
19	6.6	0.704	0.707	0.021
20	7.6	0.759	0.836	0.025
21	6.0	0.701	0.671	0.020
22	6.6	0.740	0.791	0.023
23	9.2	0.786	0.734	0.020**
24	9.2	0.780	0.806	0.023
25	9.0	0.787	0.762	0.021
26	7.4	0.739	0.645	0.017**
27	8.0	0.749	0.722	0.020
28	6.4	0.718	0.785	0.024
29	6.8	0.750	0.761	0.022***
30	7.0	0.715	0.771	0.024
31	7.2	0.761	0.771	0.022
32	7.2	0.746	0.734	0.021**
Mean	8.0	0.762	0.763	0.022

$^\dagger$  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. thyrsoidea* 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 ( $r = 0.263$ ;  $P = 0.052$ ; 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. thyrsoidea* (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

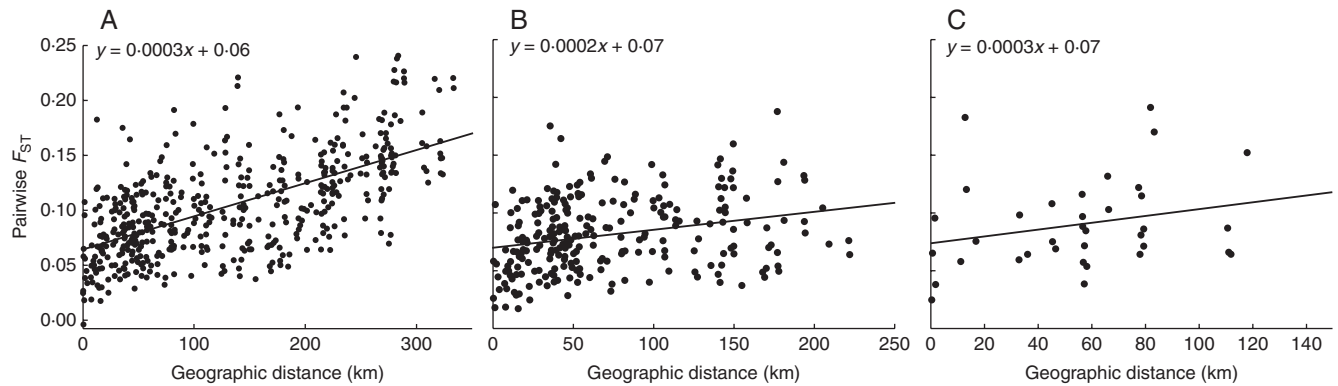


FIG. 2. Pairwise genetic distance ( $F_{ST}$ ) and geographical distances (km) between populations of *Campanula thyrsooides*: (A) all studied populations, (B) populations in the eastern region (numbers 10–32) and (C) populations in the western region (numbers 1–9).

bottlenecks were found despite the species rareness and isolation of some of its populations.

Population size did not affect molecular diversity in *C. thyrsooides* 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. thyrsooides* 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. thyrsooides*, the longevity of individuals despite its monogamy, 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. thyrsooides* 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. thyrsooides* is predominantly outbreeding (Ægisdóttir *et al.*, 2007a) with low inbreeding coefficient ( $F_{IS} = 0.022$ ), we suggest that the breeding system of *C. thyrsooides* 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 ( $F_{IS}$ ) was low in all populations (average  $F_{IS} = 0.022$ ), 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. thyrsooides* 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. thyrsooides* in this study showed rather low values of 0.10 for  $F_{ST}$  and 0.12 for  $G_{ST}$ . It has, however, been argued that the high levels of within-population heterozygosity usually found with microsatellites yield low differentiation indices among populations

TABLE 3. Summary of analysis of molecular variance (AMOVA) of 32 populations of *Campanula thyrsooides* grouped in two regions (west versus east)

Source of variation	Variance component			P
	d.f.	Absolute	%	
Among regions	1	0.11	5.39	<0.001
Among populations	30	0.16	7.91	<0.001
Within regions, within populations	1440	1.73	86.7	<0.001
Total	1471			

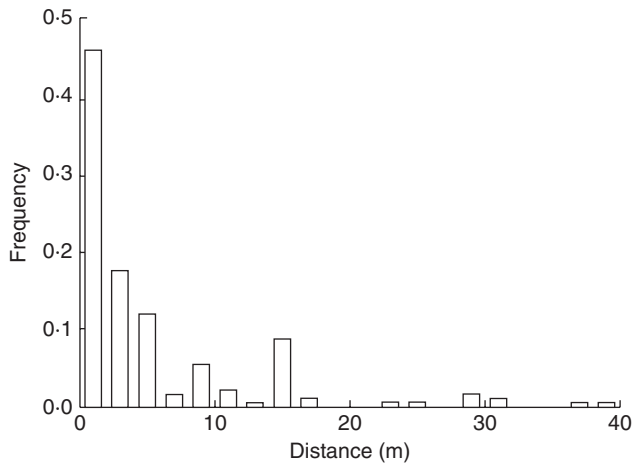


FIG. 3. Frequency of pollen movement distances (m) between *Campanula thyrsooides* plants on the Furka Pass in the Swiss Alps, as indicated by fluorescent powder traces in summer 2004 and 2005 ( $n = 173$ ).

(Hedrick, 1999, 2005), and that a standardized measure of genetic differentiation ( $G'_{ST}$ ) should routinely be reported for microsatellite data (Heller and Siegismund, 2009). Using  $G'_{ST}$ , a considerable genetic differentiation (0.53) was observed as could be expected for a rare alpine monocarpic species with limited seed dispersal capacities (Loveless and Hamrick, 1984). The value of  $G'_{ST}$  which is about four times higher compared with  $G_{ST}$  in the present study is in accordance with other comparisons of  $G'_{ST}$  and  $G_{ST}$  (see table S1 in Heller and Siegismund, 2009). Gene flow between the *C. thyrsooides* populations in the present study is therefore clearly restricted and occurs mostly among close and adjacent populations (see Fig. 2A) as the correlation between geographic distance and genetic distance suggests (Wright, 1943). The restricted gene flow between the studied populations was to be expected. The Alpine landscape with high mountain ridges and steep valleys may limit or even block gene flow among plant populations (Cain *et al.*, 2000; Theurillat and Guisan, 2001). This may result in a stronger genetic differentiation among populations than in more homogenous landscapes (Till-Bottraud and Gaudeul, 2002). Therefore, the considerable population differentiation even among populations that are geographically not very distant (Fig. 2) may relate to the particularities of the landscape in the Alps. In this context, a combination of genetic information with fine-resolution qualitative landscape data appears to be a promising future approach (Holderegger and Wagner, 2008).

#### Genetic differentiation among regions

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. thyrsooides*, 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. thyrsooides* 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 thyrsooides* 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. thyrsooides* 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. thyrsooides*, 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 ( $\pm$  s.d.) of  $\ln$  probability of data [ $\ln P(D)$ ] for  $K$  from 1 to 5 as implemented in STRUCTURE on *Campanula thyrsoidea*.

