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Spatial isolation and genetic differentiation in naturally fragmented plant populations of the Swiss Alps

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Abstract

Aims

The effect of anthropogenic landscape fragmentation on the genetic diversity and adaptive potential of plant populations is a major issue in conservation biology. However, little is known about the partitioning of genetic diversity in alpine species, which occur in naturally fragmented habitats. Here, we investigate molecular patterns of three alpine plants (*Epilobium fleischeri*, *Geum reptans* and *Campanula thyrsoides*) across Switzerland and ask whether spatial isolation has led to high levels of population differentiation, increasing over distance, and a decrease of within-population variability. We further hypothesize that the contrasting potential for long-distance dispersal (LDD) of seed in these species will considerably influence and explain diversity partitioning.

Methods

For each study species, we sampled 20–23 individuals from each of 20–32 populations across entire Switzerland. We applied Random Amplified Polymorphic Dimorphism markers to assess genetic diversity within (Nei's expected heterozygosity, H_e; percentage of polymorphic bands, P_p) and among (analysis of molecular variance, Φ_{st}) populations and correlated population size and altitude with within-population diversity. Spatial patterns of genetic relatedness were investigated using Mantel tests and standardized major axis regression as well as unweighted pair group method with arithmetic mean cluster analyses and Monmonier's algorithm. To avoid known biases, we standardized the numbers of populations, individuals and markers using multiple random reductions. We modelled LDD with a high alpine wind data set using the terminal velocity and height of seed release as key parameters. Additionally, we assessed a number of important life-history traits and factors that potentially influence genetic diversity partitioning (e.g. breeding system, longevity and population size).

Important findings

For all three species, we found a significant isolation-by-distance relationship but only a moderately high differentiation among populations (Φ_{st}: 22.7, 14.8 and 16.8%, for *E. fleischeri*, *G. reptans* and *C. thyrsoides*, respectively). Within-population diversity (H_e: 0.19–0.21, P_p: 62–75%) was not reduced in comparison to known results from lowland species and even small populations with <50 reproductive individuals contained high levels of genetic diversity. We further found no indication that a high long-distance seed dispersal potential enhances genetic connectivity among populations. Gene flow seems to have a strong stochastic component causing large dissimilarity between population pairs irrespective of the spatial distance. Our results suggest that other life-history traits, especially the breeding system, may play an important role in genetic diversity partitioning. We conclude that spatial isolation in the alpine environment has a strong influence on population relatedness but that a number of factors can considerably influence the strength of this relationship.

Keywords: *Campanula thyrsoides* • *Epilobium fleischeri* • *Geum reptans* • isolation by distance • molecular diversity

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INTRODUCTION

The effect of landscape fragmentation on the genetic diversity of plant populations is a major issue in conservation biology (Frankham *et al.* 2002; Young *et al.* 1996). It is important to predict a species' extinction risk as a result of habitat loss and impeded genetic connectivity between populations in order to establish suitable protection measures (Gilpin and Soulé 1986). This is particularly true in the rapidly changing modern landscape that is shaped by anthropogenic resource exploration such as agricultural practices, deforestation or infrastructure building (e.g. Fischer and Stöcklin 1997; Groom and Schumaker 1993). By contrast, natural fragmentation is a characteristic feature of the alpine environment due to a pronounced mountainous topography and associated abiotic heterogeneity and has played a key role in the evolution of species (Körner 2003). Alpine and subalpine plants are organized into local populations of different sizes, highly structured in space and with a high capacity for extended local persistence due to perennity and/or clonality (Bliss 1971; Körner 2003). On the other hand, colonization of new sites is a slow and irregular process, which largely depends on rare long-distance dispersal (LDD) events (Austrheim and Eriksson 2001). While a growing number of studies have evaluated the genetic consequences of habitat fragmentation in the lowlands (Bacles *et al.* 2004; Bartish *et al.* 1999; Buza *et al.* 2000; Galeuchet *et al.* 2005; Hensen *et al.* 2005; Pluess and Stöcklin 2004a; Rosquist and Prentice 2000), the effect of spatial isolation on alpine species is poorly known. In the light of global warming, alpine plants are particularly vulnerable to rapid change (Pauli *et al.* 2003) and it is therefore important to estimate their adaptive potential.

In general, habitat fragmentation and the resulting decline in population size can have a multitude of effects, such as erosion of genetic variation, increased random genetic drift or elevated inbreeding, which can all enhance the risk of extinction (e.g. Frankham and Ralls 1998; Gilpin and Soulé 1986; Young *et al.* 1996). An intuitive consequence of spatial isolation is a reduced genetic connectivity between populations which leads to stronger dissimilarity of population pairs with increasing distances, generally referred to as 'isolation by distance' (IBD; Wright 1943). However, the magnitude and significance of IBD patterns are often considered to be a result of a number of additional factors, such as physical barriers, dispersal ability, effective population size, maximum geographic sampling distance or time since colonization (e.g. Crispo and Hendry 2005; Garnier *et al.* 2004), so that general predictions are difficult to make. Further, irrespective of habitat fragmentation, there is still considerable uncertainty about the relative influence of specific environmental constraints, the Quaternary history and life-history traits on the genetic diversity of a given species. Since molecular markers have different resolutions and modes of inheritance (Lowe *et al.* 2004), they tend to emphasize different factors. In this respect, reviews of nuclear marker studies of predominantly lowland species showed that long-lived, outcrossing, late successional plant species retain the greatest share of their genetic variability within populations, while for annual, selfing and/or early successional taxa, a high percentage of genetic diversity is found among populations (Hamrick and Godt 1989; Nybom 2004; Nybom and Bartish 2000). Similar results have been shown for alpine species (Till-Bottraud and Gaudeul 2002) but, with only a few studies available, an effect of harsh alpine habitats cannot be ruled out. On the other hand, a meta-analysis of chloropolast DNA studies found little influence of life-history traits on genetic diversity, but evidence for glaciation-derived patterns (Aguinagalde *et al.* 2005). Problematic today for a general prediction of the effect of alpine landscape on the genetic diversity of plant populations is the scarcity of data from the few more or less continuously distributed plant species of alpine or arctic environments. So far, only the European alpine grasses *Poa alpina* (Rudmann-Maurer *et al.* 2007) and *Sesleria albicans* (Reisch *et al.* 2002) have been studied genetically but unfortunately these studies do not provide sufficient comparable genetic baseline data since the sampling design (e.g. low number of individuals per population and low representation in the Alps) or use of molecular markers (microsatellites versus RAPDs) impedes valid comparison.

Here, we study and compare genetic diversity and differentiation of three perennial plant species of the Swiss Alps. Given the complex interactions involved in the creation of molecular patterns as outlined above, we took particular care to standardize as many parameters as possible in order to minimize known biases (Lowe *et al.* 2004; Nybom and Bartish 2000). We standardized the number of populations, individuals, RAPD primers as well as loci for each species and further restricted the data analysis to the same maximum geographic distance within a single prominent area of post-glacial migration (Schönswetter *et al.* 2005). We decided to reanalyse results of a previous study on the alpine *Geum reptans* (Pluess and Stöcklin 2004b) with two new investigations on *Epilobium fleischeri* and *Campanula thyrsoides*. Thus, in this common framework, we can considerably improve the comparability of individual patterns. Our objective is to elucidate the effect of natural fragmentation on the genetic diversity of these three plant species in which spatial isolation can be assumed to have existed for centuries or millenia. In particular (i) we expect genetic population differentiation to be high and significantly increasing with increasing distances. Since our study species differ particularly with respect to long-distance seed dispersal, (ii) we expect relatively lower genetic differentiation and the least pronounced IBD pattern for species with morphological adaptations to seed dispersal compared to plants lacking those functional structures. (iii) We further investigate levels of within-population diversity and expect a significant decrease of diversity with decreasing population sizes.

MATERIALS AND METHODS

The plant species

Epilobium fleischeri Hochst. (Onagraceae), *Geum reptans* L. (Rosaceae) and *Campanula thyrsoides* L. (Campanulaceae) are

widespread subalpine–alpine plant species native to the European Alps and, partly, to adjacent mountain ranges in the east (Carpathians and Dinarians) and north-west (Jura; see Hegi 1995). Throughout their ranges, plants are rare but locally abundant with population sizes ranging from a few hundred to >100 000 individuals. *Epilobium fleischeri* and *G. reptans* are characteristic plants of glacier forelands appearing within few years after ice retreat. *Campanula thyrsoides* is found in mesic subalpine–alpine meadows on calcareous soil. Habitats for the three species are patchily distributed in the Alps with geographic distances among populations around 5–30 km (for habitat and population distribution maps of *E. fleischeri* and *G. reptans*, see Gerber *et al.* 1998; for *C. thyrsoides*, see Kuss *et al.* 2007). The species investigated differ with respect to several important life-history traits that potentially and differentially influence genetic diversity partitioning within and among populations (Table 1). Life-history information is accumulated from a number of literature sources or from additional unpublished experiments and observations made by the authors. Data for long-distance seed dispersal were generated with the software PAPPUS implementing an alpine wind data set and the terminal velocity of the seeds (Tackenberg 2003; Tackenberg and Stöcklin 2008). Pollen flow observations are minimum distance estimates derived from flower to flower fluorescent powder transport by mainly bumblebees (*E. fleischeri*: J. Stöcklin, unpublished results; *C. thyrsoides*: Ægisdóttir *et al.* 2007) or flies (*G. reptans*: Pluess and Stöcklin 2004b). Breeding system data are derived from pollinator exclosure and manual crossing experiments (*E. fleischeri*: Theurillat 1979; *C. thyrsoides*: Ægisdóttir *et al.* 2007; *G. reptans*: Rusterholz *et al.* 1993). Population size was estimated by counting flowering individuals in the confined geographic area or, if individuals >250, by counting a subset of the individuals and extrapolating to the entire occupied area. Ramet age estimates stem from herb chronology studies of roots with a representative number of individuals as presented in Dietz and Ullmann (1998). It would be desirable to have information on the potential genet age in the clonal species *E. fleischeri* and *G. reptans*, but investigations are still missing.

Sampling design

For all three species, we sampled a minimum of 20 individuals per population and a minimum of 20 sites spread over the Swiss Alps. Leaf materials from randomly chosen individuals within a population were sampled, dried with silica gel and stored at room temperature until analysis. To avoid resampling the same clone in *E. fleischeri* and *G. reptans*, a minimum distance of 4 m was chosen. Care was taken to cover the same altitudinal range and a similar geographic pattern wherever practical. In the case of *C. thyrsoides*, we extended the sampling to additional populations in order to test the robustness of genetic pattern through randomization procedures (see below). Location of sampling sites and population descriptions are summarized in Fig. 1 and Supplementary Data, Appendix S1 (for detailed information on *G. reptans*, see Pluess and Stöcklin 2004b).

RAPD markers

DNA extraction and quantification procedures for *E. fleischeri* and *G. reptans* are identical and are described in Pluess and Stöcklin (2004b). For DNA extraction of *C. thyrsoides*, we used a DNeasy Plant 96 Kit (Qiagen, Hilden, Germany) and included 25 mg polyvinylpyrrolidone (Fluka, Buchs, Switzerland) to each sample to remove polyphenols in the first extraction step. DNA quantification was done with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). After an initial screening of up to 60-decamer primers, we restricted the final analysis to five primers for each species rendering 47–89 scorable polymorphic loci (Kit A, K and P, Operon Technologies Inc., Alameda, CA, USA and M-6 Microsynth, Balgach, Switzerland). We attempted to use the same five primers for all species but polymerase chain reaction (PCR) products could not be obtained with this prerequisite. Therefore, we selected the primers with the highest number of reproducible polymorphic bands: *E. fleischeri* (OPA-8 [GTGACGTAGG],

Table 1: life-history traits of three alpine plant species

Species	Habitat[a]	Altitudinal range (m)[a]	Breeding system	Seeds/ population (Mio)	Dispersal (% >1 km)[b]	Pollen flow (m)	Clonality	Ramet age (years)
Epilobium fleischeri	Glacier forelands, river bank	1 000–2 700	Mixed[c]	4.5[d]	Wind (0.5)	Insects (30[e])	Rhizomatous[c]	30[d]
Geum reptans	Glacier forelands, blockfields	1 950–3 500	Outcrossing[f]	10[g]	Wind (0.005[g])	Insects (30[g])	Stolons[g]	30[g]
Campanula thyrsoides	Alpine meadows, pastures	1 300–2 800	Outcrossing[h]	1.5[h]	Wind (0.001)	Insects (39[h])	Non-clonal[a]	3–16[i]

[a]Hegi (1995).
[b]O. Tackenberg (personal communication).
[c]Theurillat (1979).
[d]Stöcklin and Bäumler (1996).
[e]Stöcklin (unpublished results).
[f]Rusterholz *et al.* (1993).
[g]Pluess and Stöcklin (2004b).
[h]Ægisdóttir *et al.* (2007).
[i](Kuss *et al.* 2007).

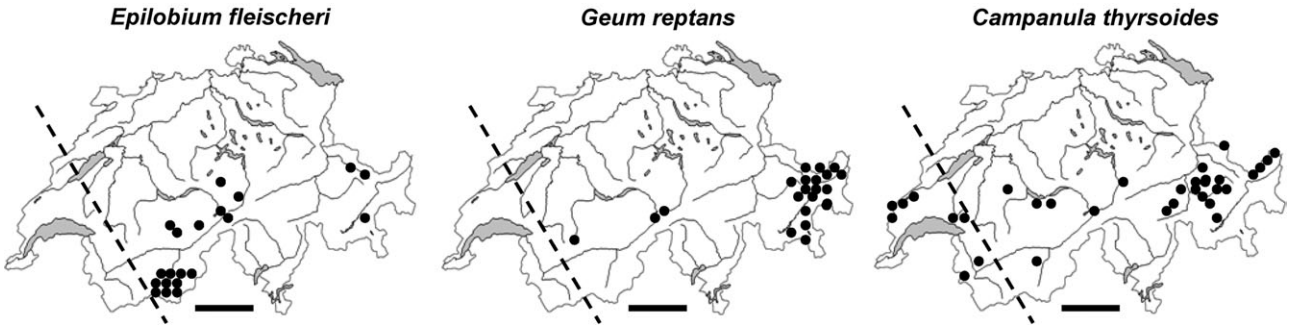

Figure 1: Geographic distribution of the studied populations of *Epilobium fleischeri, Geum reptans* and *Campanula thyrsoides* in the Swiss Alps and the Jura Mountains. Dashed line represents approximate borderline between two glacial refugia (Schönswetter *et al.* 2005). Bar: 50 km. Map for *G. reptans* modified from Pluess and Stöcklin (2004b).

OPA-9 [GGGTAACGCC], OPA-12 [TCGGCGATAG], OPA-15 [TTCCGAACCC] and OPP-12 [AAGGGCGAGT]); *C. thyrsoides* (OPA-7 [GAAACGGGTG], OPA-11 [CAATCGCCGT], OPA-13 [CAGCACCCAC], OPA-19 [CAAACGTCGG] and OPP-3 [CTGATACGCC]) and *G. reptans* (OPP-8 [ACATCGCCCA], OPP-9 [GTGGTCCGCA], OPP-17 [TGACCCGCCT], OPP-19 [GGGAAGGACA] and M-6 [GTGGGCTGAC]). Amplifications were carried out in 25-µl reaction mixture containing 3 ng of template DNA, 100 µM dNTPs, 0.2 µmol/l primer, 1 × *Taq* Polymerase Buffer (Qiagen); additional 0.5 mmol/l $MgCl_2$ for the primers OPA-12, OPP-17, OPP-19 and M-6; additional 1.5 mmol/l $MgCl_2$ for the primers OPA-8, OPA-15 and 1 U *Taq* DNA Polymerase (Qiagen). For primers and amplification specifications used for *G. reptans*, see Pluess and Stöcklin (2004b). To assure consistency in the PCRs, we kept aliquots of a single master mix per two primers for all samples only adding primer, *Taq* Polymerase and DNA before PCR. All PCRs were performed in the same thermal cycler (PTC-100, MJ Research, Inc., Watertown, MA, USA) programmed for 60 s at 93°C to denature the DNA followed by 34 cycles of 30 s at 92°C, 30 s at 37°C and 90 s at 72°C. Final extension lasted for 5 min at 72°C. Samples were kept at 4°C until further analysis. The PCR products were separated on 1.6% agarose gels (Sea Kem LE agarose, BMA, Rockland, MD, USA) in 1 × Tris–acetate–ethylene diamine tetraacetic acid buffer in an electrical field (170 mV). Depending on the RAPD primer, gels were run between 1.75 and 2.5 h and stained with ethidium bromide for 20 min. We visualized the banding pattern under ultraviolet light and scored the presence and absence of bands within an estimated fragment length range of 450–2 000 bp from digital images (AlphaDigiDoc and AlphaEaseFC software, Alpha Innotech Corp., San Leandro, CA, USA).

We repeated amplification at timely intervals with 12 initial screening samples, i.e. three individuals from each of four distinct populations in order to assure reproducibility and assess genotyping errors (Bonin *et al.* 2004). This was also repeatedly done for randomly chosen individuals. All amplifications contained blind samples (no DNA) as well as foreign DNA from different plant species (*Campanula barbata*, *Senecio incanus* and *Hypochaeris uniflora*). Monomorphic bands served as references for genotype errors within and between amplification. Only bands that could be scored unambiguously were used for the statistical analysis. The final presence/absence data matrix contained for *E. fleischeri* 400 individuals from 20 populations, for *G. reptans* 386 individuals from 20 populations and for *C. thyrsoides* 736 individuals from 32 populations (Supplementary Data, Appendix S1).

Statistical analysis

In order to achieve a comparable framework for the statistical analysis, we adjusted our data sets in several consecutive steps to avoid biased results. First, we restricted the presence/absence matrix to bands whose observed frequencies were $<1 - (3/N)$, where N is the mean number of sampled individuals per population (Lynch and Milligan 1994). Second, in an initial analysis, we visualized the molecular indices with the software 'Barrier' (Manni *et al.* 2004) in order to detect patterns of molecular contrast that geographically coincide with borders of proposed post-glacial migration areas (Schönswetter *et al.* 2005). In such a case, we restricted the analysis to populations within the same area avoiding trans-border effects. Third, for the calculation of diversity and differentiation measures that are valid for interspecies comparisons, we matched the number of populations, individuals and loci for each species through multiple random reductions of the parameters (100 subsamples) similar to the approach of Leberg (2002). Even though the information on the heterozygosity of populations was lacking, we assume that Hardy–Weinberg equilibrium was not violated. Pollination experiments in *G. reptans* and *C. thyrsoides* showed that both species are obligatory outbreeders with low seed set after self-pollination and no subsequent germination (Ægisdóttir *et al.* 2007; Rusterholz *et al.* 1993). *Epilobium fleischeri* is known to be largely outbreeding but having the potential for selfing (Theurillat 1979; Stöcklin, unpublished results). For this species, repeated calculations with varying F_{is} from 0 to 1 at 0.25 step intervals increased analysis of molecular variance (AMOVA)-derived Φ_{st} values but maximum increase was <1%. All statistical analyses were

restricted to polymorphic bands and all computing was performed in '*R*' (Ihaka and Gentleman 1996) using the *R*-libraries 'ade4' (Thioulouse *et al.* 1997), 'vegan' (Dixon 2003), 'smatr' (Warton *et al.* 2005) and self-written code.

Two commonly used indices of molecular diversity within populations were calculated: (i) Nei's expected heterozygosity H_e (Nei 1978) and (ii) the percentage of polymorphic bands (P_p). To quantify the variation of molecular diversity among populations, we calculated the coefficient of variance for H_e and P_p and compared species-specific indices with univariate analysis of variance (ANOVA) and Tukey HSD (Honestly Significant Difference) test. For each species, we used Pearson correlation statistics to assess the correlation between H_e and P_p as well as between population sizes and molecular indices. In cases with skewed population size distributions, we applied Spearman's rank correlation. Moreover, the relation between altitude as well as population size and molecular diversity was assessed as a linear regression. Population differentiation, or among-population diversity, was calculated using AMOVA-derived fixation index Φ_{st} (Excoffier *et al.* 1992). The species-specific variance of Φ_{st} values, obtained from multiple random reduction subsampling (see above), was then compared with a univariate ANOVA.

To test for IBD (Slatkin 1987), we applied Mantel test statistics correlating the genetic distance matrix (pairwise Φ_{st} values) and the geographic distance matrix (Euclidean square distances). Significance levels were obtained after performing 10 100 and 10 000 random permutations for the pairwise genetic distances (Φ_{st}) and the Mantel test, respectively. We used standardized major axis (SMA) regression to quantify the pattern of linear covariation (Rousset 1997) and compared species-specific regression slopes and 95% confidence intervals using one-sample tests with bootstrapping (n = 10 000) over independent population pairs as implemented in 'smatr' (Warton *et al.* 2005).

Further, we calculated an unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of pairwise Nei's unbiased genetic distances (Nei 1978) to test for spatial separation and displayed the results as dendrograms. Stable clusters were indicated (*) according to the 50% majority rule (Lowe *et al.* 2004) after bootstrapping of 10 000 replicates.

RESULTS

The adjustment of the presence/absence matrices following Lynch and Milligan (1994) resulted in 52 of 64 polymorphic loci in *E. fleischeri*, 49 of 51 in *G. reptans* and 47 of 53 in *C. thyrsoides*. None of the scored bands were fixed at the population level. Identical RAPD phenotypes were found twice for the clonal *E. fleischeri* originating from two distinct populations of the Scaletta glacier forefield and restricting the data set to 398 instead of 400 phenotypes. Similarly, identical phenotypes occurred in two populations of the clonal *G. reptans* sampled in different glacier forefields (384 instead of 386 phenotypes). In *C. thyrsoides*, all 736 phenotypes were different. For interspecies comparisons, we excluded those eight populations of *C. thyrsoides* that belong to a separate area of post-glacial migration (Supplementary Data, Appendix S1: populations 1–8). Final calculations were then based on 47 marker bands (randomly chosen for *E. fleischeri* and *G. reptans*), 20 populations for each species (randomly chosen for *C. thyrsoides*) and 20 individuals per population.

Molecular diversity within populations

Mean genetic diversities, H_e, were similar in all species (*E. fleischer*: H_e = 0.19, *G. reptans*: H_e = 0.21 and *C. thyrsoides*: H_e = 0.20) and only significantly different between *G. reptans* and *E. fleischeri* ($P < 0.01$).

The percentage of polymorphic loci, P_p, was highest for *E. fleischeri* (P_p = 74.8%), intermediate for *G. reptans* (P_p = 70.1%) and lowest for *C. thyrsoides* (P_p = 61.8%). Two of the three species were significantly different from each other (*E. fleischeri* and *C. thyrsoides*: $P < 0.01$; *G. reptans* and *C. thyrsoides*: $P < 0.01$), while no difference was found between *G. reptans* and *E. fleischeri* ($P > 0.05$). Summary statistics for species-specific diversity indices are presented in Table 2; the population-specific indices are listed in Supplementary Data, Appendix S1. In all three species, H_e and P_p were positively correlated (*E. fleischeri*: $R = 0.74$, $P < 0.001$; *G. reptans*: $R = 0.70$, $P < 0.001$; *C. thyrsoides*: $R = 0.46$, $P < 0.01$). In general, within-population measures of *G. reptans* based on 47 marker bands (this study) were similar or identical to the results based on 49 loci (Pluess and Stöcklin 2004b). Furthermore, we detected no influence of population size on the molecular diversity of *G. reptans* (H_e: $R = 0.26$, $P = 0.27$; P_p: $R = -0.01$, $P = 0.98$) or *C. thyrsoides* (H_e: $R = 0.30$, $P = 0.20$; P_p: $R = 0.05$, $P = 0.84$). By contrast, for *E. fleischeri*, genetic diversity significantly decreased with increasing population size (H_e: $R = -0.6$, $P = 0.01$; P_p: $R = -0.52$, $P = 0.02$). However, this relationship was considerably influenced by the two largest populations with >1 00 000 individuals and in a non-parametric Spearman correlation, this relationship was not significant anymore (H_e: $R = -0.24$, $P = 0.30$; P_p: $R = -0.06$, $P = 0.80$). Molecular diversity was not related to altitude, except for a single significant increase of P_p with increasing altitude in *E. fleischeri* (*E. fleischeri*—H_e: $R = 0.25$, $P = 0.1$; P_p: $R = 0.19$, $P = 0.03$; *G. reptans*—H_e: $R = 0.1$, $P = 0.1$; P_p: $R = 0.01$, $P = 0.3$ and *C. thyrsoides*—H_e: $R = -0.04$, $P = 0.66$; P_p: $R = -0.05$, $P = 0.86$).

Spatial differentiation

Among-population diversity indices were significantly different among the three species ($P < 0.001$) with *E. fleischeri* showing highest population differentiation (Φ_{st} = 22.7%) and *G. reptans* (Φ_{st} = 14.8%) and *C. tyrsoides* (Φ_{st} = 16.8%) lower differentiation levels (for summary statistics see Table 2). In *E. fleischeri*, all pairwise Φ_{st} values were significantly different, while one population pair in each of *G. reptans* or *C. thyrsoides* was genetically not differentiated, although separated geographically by >2 km (SCE, GR1) or 4 km (SCM, FTA), respectively.

For all three species, we found a significant IBD pattern as calculated with Mantel test statistics (Fig. 2, Table 2; *E. fleischeri*: $R = 0.57$, $P < 0.001$; *G. reptans*: $R = 0.81$, $P < 0.001$

Table 2: molecular diversity and differentiation indices of three alpine plant species

	Genetic diversity within populations		Genetic diversity among populations	
	H_e	P_p (%)	Φ_{st} (%)	IBD
Epilobium fleischeri	Mean = 0.19	Mean = 74.8	Mean = 22.7	$R = 0.57$
	Range = 0.13–0.22	Range = 59.6–86.5	Range = 20.1–24.9	$P < 0.001$
	SE = 0.006	SE = 1.4	SE = 0.098	pΦst = 4.7–44.4%
	CV = 11.8%	CV = 7.5%	CV = 4.3%	pgeo = 0.4–191.2 km
Geum reptans	Mean = 0.21	Mean = 70.1	Mean = 14.8	$R = 0.81$
	Range = 0.16–0.24	Range = 48.0–80.0	Range = 13.9–15.4	$P < 0.001$
	SE = 0.004	SE = 1.7	SE = 0.028	pΦst = 1.9–44.9%
	CV = 7.7%	CV = 9.3%	CV = 1.9%	pgeo = 0.2–208.1 km
Campanula thyrsoides	Mean = 0.20	Mean = 61.84	Mean = 16.8	$R = 0.32$
	Range = 0.18–0.22	Range = 53.2–76.6	Range = 16.3–17.3	$P = 0.007$
	SE = 0.003	SE = 1.3	SE = 0.036	pΦst = 2.3–29.3%
	CV = 6%	CV = 8.4%	CV = 2.1%	pgeo = 0.3–235.6 km

H_e, Nei's genetic diversity; P_P, percentage of polymorphic loci; Φ_{st}, AMOVA-derived fixation index Φ_{st}, standardized using multiple random reductions; CV, coefficient of variance; pΦst, pairwise Φ_{st}; pgeo, pairwise geographic distance.

and *C. thyrsoides*: $R = 0.32$, $P = 0.007$). The slope of the SMA regression lines was significantly steeper in *G. reptans* compared to the other two species (each $P < 0.001$), while no difference was found between *E. fleischeri* and *C. thyrsoides* ($P = 0.12$). The equations for the SMA including the slope- and intercept-specific standard errors are described as follows—*E. fleischeri*: $y = 0.01729\ (0.0062) + 1.025 \times 10^{-03}\ (6.159 \times 10^{-05})\ x$; *G. reptans*: $y = 0.06563\ (0.00512) + 1.536\ 10^{-03}\ (6.512 \times 10^{-05})\ x$ and *C. thyrsoides*: $y = 0.08874\ (0.00671) + 9.34\ 10^{-04}\ (6.502 \times 10^{-05})\ x$.

UPGMA cluster analysis (Fig. 3) and application of Monmonier's algorithm (results not shown) for *E. fleischeri* and *G. reptans* revealed no geographic patterns of genetic differentiation that coincide with the proposed areas of post-glacial migration in the western Alps (Schönswetter *et al.* 2005). In both species, all population pairs were significantly differentiated but stable dendrogram clusters were only present for a single geographically isolated population each (MOR and FLS, respectively). Even populations in close vicinity did not consistently group together. In *C. thyrsoides*, with both methods, we detected a clear separation of populations located in western Switzerland from those in central and eastern regions with the north-south running Aosta-Rhône-Valley as the geographic border. Within the two main UPGMA clusters (Fig. 3), stable branches were mostly formed by population pairs separated by distances <2 km. Nevertheless, two population pairs with distances of 49 and 73 km from each other formed stable ties (LAH, VAL and UNB, LAS, respectively). All population pairs were significantly differentiated.

For *C. thyrsoides*, we could further evaluate the effect of post-glacial migration on population differentiation by repeating the analyses with the whole data set of 24 + 8 populations (Fig. 3). A high proportion of variability was explained by genetic differences between the two groups of populations ($\Phi_{ct} = 10.3\%$) and the total genetic variability among populations amounted to 27.2% (Φ_{st}). Further, we found a significant IBD pattern within the 8 western populations ($R = 0.34$, $P = 0.03$), the 24 central/eastern populations ($R = 0.32$, $P < 0.001$) as well as for the total of 32 populations ($R = 0.53$, $P < 0.001$). The slopes of the SMA lines among the three regions were all significantly different ($P < 0.001$), steepest for western populations ($n = 8$), intermediate for the central/eastern ones ($n = 24$) and lowest for all populations ($n = 32$).

DISCUSSION

Spatial isolation and genetic differentiation

In all three species, we found a significant and positive IBD pattern that supports our hypothesis that genetic connectivity among populations decreases with increasing spatial distance as a result of natural fragmentation. At a distance of <200 km, population pairs in all species were highly differentiated with maximum Φ_{st} values ranging from 29% (*C. thyrsoides*) to 44% (*E. fleischeri* and *G. reptans*). In addition, Mantel plots of each species showed a considerable amount of scatter demonstrating a large variability of genetic differentiation at a given distance (Fig. 2). This is most pronounced for *E. fleischeri* where even at a distance of <5 km, Φ_{st} values ranged from 5 to 35%. Such a high variability suggests that genetic connectivity between populations has a strong stochastic component at all spatial scales and that the populations are not in gene flow/drift equilibrium (Hutchison and Templeton 1999). Apart from genetic drift, founder events during post-glacial colonization and/or bottlenecks due to demographic stochasticity may contribute to the large variability in pairwise Φ_{st} values encountered.

For *C. thyrsoides*, we found that populations belong to two different areas of post-glacial migration, so that we decided

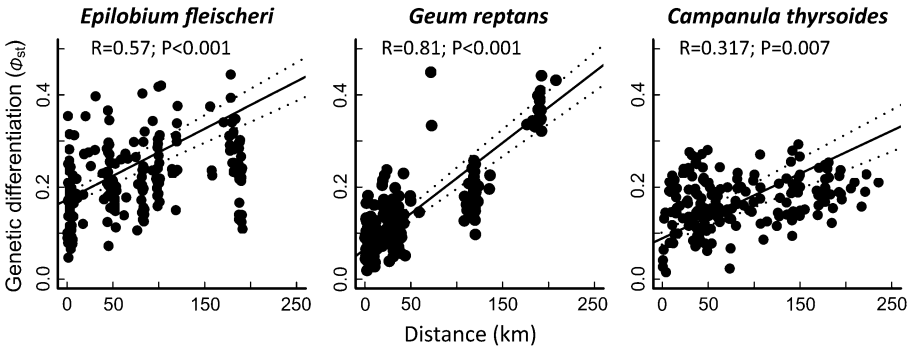

Figure 2: Matrix correlation of genetic (pairwise Φ_{st} values) and geographic distances. Solid line: SMA regression; dotted lines: SMA 95% confidence interval.

to standardize the data for among-species comparisons. However, analysing the complete data set of *C. thyrsoides* allows us to evaluate, first, the potential effect of the Quaternary history on molecular diversity patterns, and second, the bias introduced to those patterns when the effect of the Quaternary is not acknowledged. The two groups of populations are significantly differentiated with 10.3% (Φ_{ct}) of the genetic diversity partitioned between the groups. Hence, gene flow between the groups has not been strong enough during the last centuries or millennia as to mask the effect of isolation in different periglacial refugia. Within each group, we found a significant IBD pattern suggesting that recent gene flow is also impeded (see above). Trans-border analysis now shows an increase of Φ_{st} values from 16.8% (20 populations) to 27.2% (32 populations), an increasing Mantel correlation from $R = 0.32$ to $R = 0.53$, but a decreasing slope of the SMA regression line. These results clearly support our decision to standardize the species' data sets and provide indication that genetic diversity patterns in alpine species are not shaped by natural fragmentation alone.

Still, it is important to ask whether IBD is a characteristic feature of alpine plant populations and whether IBD is more pronounced under alpine compared to lowland conditions. The few studies focussing on alpine plants find inconsistent IBD relationships and the significance of an IBD pattern to be dependent on population subgrouping or, as discussed above, on geographic scale and post-glacial migration history. For example, in *Eryngium alpestris* no significant IBD was found at a geographic distance of 250 km (Gaudeul *et al.* 2000). In contrast, when subdivisions of 2 of these 14 populations were acknowledged, the overall IBD was significantly positive. For the subdivided populations within individual valleys, a positive IBD was only found at distances up to 0.2 or 2 km. Such an effect of population subdivision was not found in any of our study species (data not shown). In a study on *Rumex nivalis*, IBD was only significant within a single large region of Switzerland (the same glacial refugia investigated in the present study), but the correlation was non-significant when populations from larger distances were included (Stehlik 2002). Moreover, a significant IBD has been found in *Hypericum nummularium* for populations in the Alps, but IBD was lacking in the Pyrenees (Gaudeul 2006). As for alpine species, no clear indication of the causes governing an IBD pattern or its magnitude is visible for lowland species of fragmented or continuous populations. Presence or absence of an IBD is, again, explained by a multitude of potentially important factors, such as time since colonization (Jacquemyn *et al.* 2004), general rarity (Dittbrenner *et al.* 2005), breeding system (Irwin 2001), dispersal potential (Coleman and Abbott 2003), ocean currents (Bond *et al.* 2005) or maximum geographic distance (Hilfiker *et al.* 2004; Moyle 2006). Additionally, there are methodological implications that need to be taken into account since Mantel test statistics give only insights into the general genetic patterns within the data set while regional genetic patterns can considerably influence the overall results (e.g. Stehlik *et al.* 2001). Spatial autocorrelation methods have been used to overcome these shortcomings but calculations depend on the assignment of distance classes ideally with equal numbers of population pairs. This prerequisite is considerably hard to meet in multi-species comparisons where field population availability often counteracts the need for a single set of distance classes.

In the present study, we standardized a number of the above-mentioned factors that potentially influence IBD and we will discuss the relative role of specific life-history traits further down. It can, however, be said that in the current absence of standardized comparative studies or meta-analyses with a large number of species, there is no ample evidence that alpine species behave differently than lowland species.

Spatial isolation and within-population diversity

Our three species have similar values of mean genetic diversity (H_e = 0.19 to 0.21), which are in concordance with other alpine species such as *E. alpestris* (H_e = 0.20, Gaudeul *et al.* 2000) and *Trollius europaeus* (H_e = 0.22; Despres *et al.* 2002) or the rather wide range of H_e found in many other alpine or lowland species (see Nybom 2004; Till-Bottraud and Gaudeul 2002). The results for mean polymorphic loci are much more diverging but nevertheless high and show a considerable amount of variance (P_p = 62–75%; Table 2). It has to be borne in mind

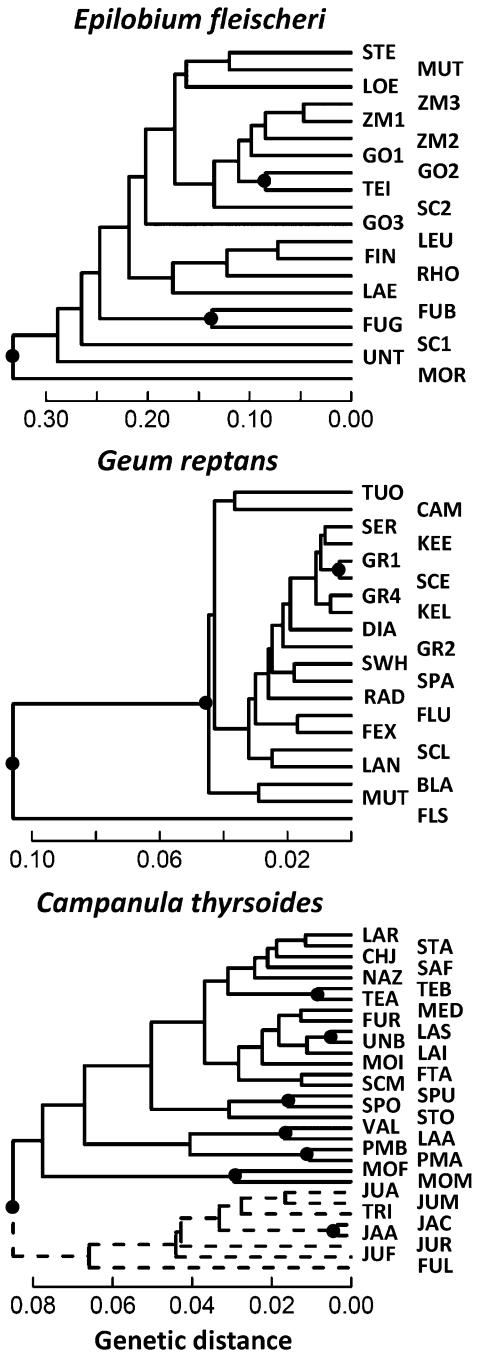

Figure 3: Dendrograms of the UPGMA cluster analysis based on Nei's (1978) unbiased measure of genetic distance (*indicate bootstrap values >50%, based on 10 000 permutations). Dashed clusters represent populations belonging to different glacial refugia.

that measures of H_e are considered less sensitive to detect consequences of isolation and population bottlenecks than alternatives such as allelic diversity (Amos and Balmford 2001). Therefore, we would expect to find an effect of fragmentation and an effect of declining population sizes with measures of P_p, rather than H_e. Particularly in species with a short generation time, low levels of P_p should be found (e.g. Till-Bottraud and Gaudeul 2002; Young *et al.* 1996). Our data does show the lowest levels of P_p in the relatively short-lived *C. thyrsoides* as compared to the long-lived, clonal *E. fleischeri* or *G. reptans*. However, levels of P_p in *C. thyrsoides* are still high and we do not find a significant correlation between population size and P_p even though a number of populations are comprised of <100 reproductive individuals. This result suggests that even small populations of *C. thyrsoides* with only 45 flowering individuals are large enough to maintain high levels of genetic diversity and, as has been shown recently, do not suffer from inbreeding depression (Ægisdóttir *et al.* 2007). Genetic diversity was also not related to altitude so that the adaptive potential to buffer consequences of global warming is likely to be similar in all populations, irrespective of their altitudinal position. The single significant increase of P_p in *E. fleischeri* with increasing altitude shows a poor correlation and can be shown to be an artefact of primer selection. In this one case, the significance of the correlation was highly influenced by only five loci of a single primer. However, correlation statistics based on the complete data set for *E. fleischeri* of 89 polymorphic loci of 10 primers showed no significant difference of P_p in relation to altitude. Forty-seven loci are generally considered to be on the low side for RAPD investigations. Therefore, we estimated the robustness of the genetic diversity indices also with the complete data sets as well as with further randomly reduced numbers of loci (<47), individuals (<20) and populations (<20). Except for the above–mentioned significant correlation in *E. fleischeri*, the results only started to deviate significantly from the reported ones after a reduction of sampling parameters by 25% (data not shown).

In general, we have no indication that natural fragmentation has led to lasting negative consequence on within-population diversity, given the high values of H_e and P_p in all three species and the absence of a population size effect. The non-intuitive decrease of genetic diversity with population size in *E. fleischeri* was considerably influenced by the two largest populations (LEU and MOR). At least for the MOR site, there is good indication that the population expanded rapidly within the last 100 years due to the pronounced retreat of the Morteratsch glacier (Maisch *et al.* 1999). This suggests that the number of flowering ramets observed represent a limited number of genets of the colonizing population. In this context, the clonal diversity of *E. fleischeri* in relation to historical processes merits further research.

Genetic differentiation and life-history traits

The strength of IBD is indicated by the slope of the SMA line that was significantly higher in *G. reptans* and not different between *E. fleischeri* and *C. thyrsoides*. From our assumption that a high LDD potential reduces the genetic difference among populations, we expected a less pronounced IBD pattern for *E. fleischeri* than for the other species. *Epilobium fleischeri*'s LDD potential was modelled to be 100–500 times higher than *G. reptans* and *C. thyrsoides* (Table 1). However, our data does not distinguish between good and poor dispersers regarding

IBD or mean Φ_{st} in a plausible way. Other life-history traits or a combination thereof may be more influential on genetic similarity of populations than seed dispersal alone. LDD by pollen is unlikely since all our study species were pollinated by bumblebees, smaller hymenoptera or flies that usually show flight activity within a range of <1 km (see Table 1; Darvill *et al.* 2004; Osborne *et al.* 1999). A life-form effect, e.g. annuals versus long-lived perennials (Nybom 2004), is also unlikely because all species are perennial taxa. Although ramet age varied among species, population persistence as well as genet persistence for clonal species can exceed more than several 100 years.

A possible explanation for our different Φ_{st} values may be found in the breeding systems. Higher levels of among-population diversity are reported in selfing species as opposed to obligatory outbreeders (Hamrick and Godt 1989; Nybom 2004; Nybom and Bartish 2000). *Epilobium fleischeri* is known to be a mixed-mating species, i.e. generally outcrossing but allowing for selfing, and should therefore tend to be more differentiated than populations of *G. reptans* or *C. thyrsoides*. Our data indicate such a relationship but a general conclusion is difficult given only three species that differ also in a number of other life-history traits. In comparison with the few studies on alpine plants that employ dominant nuclear markers, our results for the outcrossing *G. reptans* (Φ_{st} = 14.8%) and *C. thyrsoides* (Φ_{st} = 16.8%) are within the broad range of Φ_{st} values calculated for the outcrossing *Saxifraga oppositifolia* (5%, Gugerli *et al.* 1999), *Phyteuma globularifolia* (13%, Schönswetter *et al.* 2002), *T. europaeus* (16%, Despres *et al.* 2002), *Eritrichum nanum* (17%, Stehlik *et al.* 2001) and *Bupleurum stellatum* (22%, Schönswetter and Tribsch 2005). *Epilobium fleischeri* partitioned 22.7% of its genetic diversity among populations, which is lower than the mixed-mating *Galitzkya macrocarpa* (25.1%, Wesche *et al.* 2006), *Anisodus tanguticus* (33%, Zheng *et al.* 2008), *E alpestris* (42%, Gaudeul *et al.* 2000) or the selfing *Saxifraga cespitosa* (42%, Tollefsrud *et al.* 1998), so that *E. fleischeri* seems to behave like an outcrossing species. However, *E. alpestris* is adapted to exozoochory, which is assumed to lead to higher Φ_{st} values than wind dispersal (Nybom 2004). We therefore argue that a high LDD potential in *E. fleischeri* may reduce the differentiating effects of its mating system.

Unfortunately, up to date there is no study that attempted to elucidate the complex interaction of life-history traits on genetic diversity partitioning in a standardized geographic setting. In this context, it is important to note that for the above-mentioned alpine species, we did not use the 'global Φ_{st} values' of each literature source but those values associated with 'genetic differentiation among populations within regions' in which case the size of a region was similar to our study. This reduced the bias of geographic scale, which may have been the reason behind a high 'mean RAPD Φ_{st} value' of 27% for outcrossing species as listed in a review by Nybom (2004). To conclude, our three species have Φ_{st} values between 14.8 and 22.7% that demonstrate a relatively restricted differentiation of populations and thus, we cannot confirm our initial hypothesis that natural fragmentation in the alpine environment has led to a particularly high population differentiation. The biology of a species appears to have a major influence on genetic diversity partitioning and largely masks an effect of spatial isolation.

CONCLUSION

Our results indicate that natural fragmentation has led to a significant decline of relatedness between population pairs with increasing geographic distance. However, this pattern of IBD also shows a considerable amount of variation with high levels of differentiation even at small spatial scales (<5 km). This suggests that genetic connectivity of alpine plant populations has a strong stochastic component at all spatial scales and further that population similarity is not directly associated with the LDD potential of a species. Other life-history traits (e.g. breeding system) or a combination thereof may considerably influence genetic diversity partitioning in alpine plants and in this respect, alpine plants do not differ from lowland plants of fragmented or continuous populations. Also, natural fragmentation does not necessarily result in particularly high levels of mean genetic population differentiation or in a loss of genetic diversity within populations of alpine plants. Even small populations of <50 reproductive individuals can maintain comparably high levels of genetic diversity.

SUPPLEMENTARY DATA

Supplementary Data Appendix S1 is available online at *Journal of Plant Ecology* online.

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