How glacial history and environmental variation affect population differentiation and gene flow dynamics in Alpine plant species

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Prof. Dr. Martin Spiess
Dekan

Diese Dissertation widme ich meiner Familie und meinen Freunden.

Trees are poems that earth writes upon the sky.

Khalil Gibran

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Chapter 1

General introduction

This doctoral thesis

Aims and relevance

The focus of this doctoral thesis lies on the genetic diversity and local adaptation of plant species in the European Alps. It studied the consequences of glacial history and the environmental variation of the naturally fragmented Alpine landscape for neutral genetic and adaptive processes in Alpine plant species. Together with the PhD project of J.F. Scheepens this thesis is part of the larger project "How glacial history, selection and current gene flow affect Alpine plants: Population differentiation, local adaptation and demography in a fragmented landscape" under the supervision of Professor Jürg Stöcklin. It consists of two complementary parts.

In the first part, we have comprehensively studied whether and to what extent glacial history has affected the genetic population differentiation in the two Alpine plant species *Geum reptans* L. and *Campanula thyrsoides* L. at the large spatial scale of the Alps. The main hypothesis was that phylogenetic structure of populations, caused by neutral genetic processes during isolation in glacial refugia and recolonisation of different Alpine regions, has led to population differentiation in phenotypic traits. We additionally hypothesised that at least part of the phenotypic differentiation is adaptive due to the recent environmental heterogeneity.

In the second part of the thesis, we have studied the spatial genetic structure, gene flow dynamics and colonisation potential of *Campanula thyrsoides* on a mountain plateau in the Swiss Alps at small spatial scales. Here, we focused on the seed dispersal and gene flow by pollen in order to estimate the ability of the species to assure survival and persistence in space and time as well as to support conservation efforts for this rare species.

This doctoral thesis provides a conceptual scientific framework for approaching one of the central issues in plant population biology and landscape ecology (Briggs & Walters 1997; Silvertown & Antonovics 2001), namely why plant species are able to survive in the highly heterogeneous landscape of the Alps.

The presented projects in the first part of this thesis are among the first to investigate whether the phylogeographic structure due to glacial history in Alpine plant species is reflected by phenotypic population differentiation. From the perspective of preserving Alpine biodiversity, the evaluation of the consequences of climatic oscillations and drastic range changes during Quaternary history on population differentiation is particularly important in order to evaluate the adaptive potential and migration patterns in Alpine plants under future land use and climate changes. In the second part of the thesis, we linked regional with local population processes to investigate which factors are limiting the distribution of a rare Alpine plant, and thereby the thesis provides basic knowledge for an efficient conservation strategy for this species.

Study species

In this doctoral thesis, we used two Alpine plant species, *Geum reptans* and *Campanula thyrsoides*, both have the core of their distribution range in the European Alps (Aeschimann *et al.* 2004). The first is a common Alpine plant species, while the second is rarely occurring at the regional scale and therefore red-listed in the majority of the Alpine countries (Kuss *et al.* 2007). The already detailed knowledge of the morphology, reproduction and demography of these two plant species is an optimal premise for the aims of this thesis, which were outlined above.

Geum reptans

Geum reptans L. (Rosaceae) is a widespread siliceous pioneer plant occurring in glacier forelands and on moist screes between 2,000–3,000 m a.s.l. (Aeschimann *et al.* 2004). The species can reproduce clonally or sexually. *G. reptans* is insect-pollinated and its styles, which elongate during maturation, facilitate seed dispersal by wind (Pluess & Stöcklin 2005). The clonality and longevity of individuals in this species can counteract seed loss through predation by a gall midge (Weppler & Stöcklin 2006).

Campanula thyrsoides

Campanula thyrsoides L. (Campanulaceae) is a calcareous perennial plant with overlapping generations and occurs in moderately disturbed grasslands between 1,600–2,200 m a.s.l. (Kuss *et al.* 2007). Today, two subspecies, ssp. *thyrsoides* and ssp. *carniolica*, are recognised mainly differing in their morphology (Scheepens *et al.* 2011). The species is monocarpic and a demographic study revealed that delayed flowering in this plant is due to a strong dependence of flowering on plant size (Kuss *et al.* 2008b). *C. thyrsoides* is strongly self-incompatible and artificial selfing leads to loss in seed set (Ægisdóttir *et al.* 2007). Seeds lack morphological adaptations for dispersal by wind and are mostly dispersed close to the mother plant (Kuss *et al.* 2007).

Molecular methods and experiments

In the projects of this doctoral thesis, we used a combination of modern molecular tools as well as traditional field and common garden experiments. The work also includes several advanced approaches, such as landscape genetics and linear-mixed modelling, which both were only recently introduced in the field of plant ecology.

For the estimation of genetic diversity, we used molecular markers and quantitative genetic traits. Molecular markers represent variation at the DNA level. In our projects, we either used dominant (RAPDs) or co-dominant (microsatellites) markers. Both marker types are putatively neutral and therefore allow the estimation of neutral genetic diversity, since selection can be excluded (see meta-analysis in Leinonen *et al.* 2008). RAPDs and microsatellites are both highly resolving nuclear DNA (nDNA) markers and are better suited to study genetic diversity within and among populations than chloroplast DNA (cpDNA) markers, which have only a limited geographic resolution (Schaal *et al.* 1998). Quantitative genetic traits are plant traits with a polygenic inheritance and are determined by environmental and genetic factors (Falconer 1981). For distinguishing environmental from genetic effects, common garden experiments can be used. In common garden experiments, plants are cultivated under controlled environmental conditions and thus differentiation of phenotypes grown in a common garden is due to the genetic component or maternal effects (Briggs & Walters 1997; Weiner *et al.* 1997).

Gene flow by pollen and seed dispersal can be measured indirectly from the degree of genetic differentiation among populations, but in this case, historical and contemporary gene flow cannot be distinguished. Therefore, contemporary gene flow can be measured either indirectly by means of molecular markers over one generation or directly with pollen fluorescent experiments in the field (Van Rossum *et al.* 2011). Here, we used both mentioned approaches to study the movement of pollen and seeds. In addition, we established a seed sowing experiment (Turnbull *et al.* 2000) in the field to investigate the factors limiting the distribution of plants.

Funding of the projects

The projects presented in this doctoral thesis were supported financially by a grant from the Swiss National Science Foundation (project no. 3100AO-116785) to Jürg Stöcklin, by grants from the Freiwillige Akademische Gesellschaft to Eva S. Frei and J.F. Scheepens, and by a grant from the Basler Stiftung für Biologische Forschung to Eva S. Frei. Further financial support was received from the Stiftung Dr. Joachim de Giacomi of the Swiss Academy of Sciences and by the company Jungfraubahnen Schynige Platte (Hans Zurbuchen), all to Eva S. Frei. Finally, the University of Basel has supported financially the participation at international conferences in Chandolin, Giessen, Luxembourg and Nijemegen.

Scientific background

Glacial survival in the Alps – a phylogeographic perspective

The fate of the Alpine flora during Quaternary glaciation in the European Alps is an aspect that received particular attention in historical biogeography (Hewitt 2000). In the following text “Alpine” will be used for habitats and mountain plants above the treeline in the European Alps, while “alpine” refers to the corresponding vegetation belt in other mountain ecosystems.

Generally, two hypotheses on the glacial survival of Alpine plant species have been proposed (Gugerli & Holderegger 2001). During the last Ice Age, a thick ice shield covered most mountains in the Alps. The *nunatak* hypothesis assumes that Alpine plants survived on different ice-free areas (i.e. high-Alpine nunataks) within the Alps. In contrast, according to the *tabula rasa* hypothesis, plants did not survive within the Alps, but recolonised them out of peripheral refugia after the end of the last Ice Age, approximately 10,000 years ago. Schönswetter *et al.* (2005) showed that for most common plants the *tabula rasa* hypothesis is more likely (but see Stehlik *et al.* 2001). For some Alpine plant species only a single glacial refugium has been found and recolonisation has taken place as a ‘stepping stone process’ from the Eastern to the Western Alps (Mraz *et al.* 2007).

Biogeographic studies mainly used fossil evidence and distribution patterns to investigate the impact of post-glacial migration on the diversity of Alpine plants (e.g. Merxmüller 1952). However, in the last decade, such studies have been fostered by the development of molecular markers. Molecular phylogeographic studies demonstrated that glacial history had also an impact on the population genetic structure in Alpine plant species. Based on molecular and palaeo-environmental data, Schönswetter *et al.* (2005) suggested several glacial refugia for plants at the border of the Alps (I–VII in Fig. 1). The three biogeographic break lines (A–C in Fig. 1) splitting the Alps into four major biogeographic regions were recently confirmed by phylogeographic break zones in a large molecular project including plant species from the Alps and Carpathians (Thiel-Egenter *et al.* 2011).

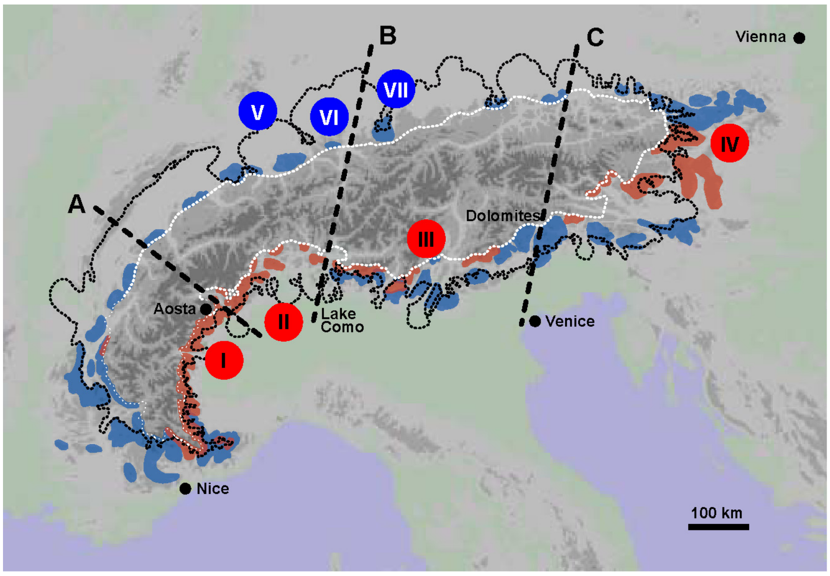

Fig. 1. Synopsis map of glacial refugia of Alpine plants in the European Alps during the last glacial maximum (ca 20,000 years ago) based on palaeogeological and molecular data. Potential peripheral refugia of siliceous (blue areas) and calcareous species (red areas) are deduced from palaeontological data. Different numbers (I–VII) indicate the glacial refugia deduced from molecular phylogeographic studies. The white broken line shows the glacial snow line, the small black broken line indicates the maximum extent of the ice sheet, and the three thick black broken lines (A–C) refer to the three break lines inferred from biogeographic studies. The map is modified from Schönswetter *et al.* (2005).

Population differentiation in Alpine plants

Random genetic drift and gene flow by pollen and seed dispersal are the main evolutionary forces causing neutral genetic differentiation in plant species (Slatkin 1977). Therefore, the degree of landscape fragmentation is one important factor that influences within-population and among-population genetic diversity (i.e. genetic differentiation) in plants because the spatial isolation of habitats may limit gene flow and thereby increase population differentiation by drift (Young *et al.* 1996). In the Alps, suitable habitats for plants are often spatially isolated because of the steep topography (Körner 2003). Indeed, the spatial isolation of Alpine habitats had a strong impact on genetic differentiation in several plant species (Kuss *et al.* 2008a). However, the species' biology (e.g. breeding system, dispersal mode) is also a major factor driving genetic diversity in plants (Nybom 2004). Therefore, the high diversity in outcrossing and wind-dispersed Alpine plants (Thiel-Egenter *et al.* 2009) may allow survival despite isolation of populations in the naturally fragmented Alpine landscape. As mentioned in the section above, an additional factor for genetic differentiation among present-day populations in Alpine plants is Quaternary history. It is assumed that gene flow was not strong enough to override effects of neutral drift and subsequent limited gene flow during isolation in different glacial refugia and recolonisation of the Alps (Schönswetter *et al.* 2005).

In the absence of drift and gene flow, divergent selection may lead to local adaptation in plant populations (Kawecki & Ebert 2004). Local adaptation is the ability of a species to alter its phenotype in response to environmental change, either in long-term (i.e. adaptive potential) or in short-term (i.e. phenotypic plasticity; Valladares *et al.* 2006). Both of these adaptive responses of the phenotype of a species are determined by its genotype. Therefore, the particularly high temporal and spatial heterogeneity of environmental conditions in Alpine habitats may cause adaptive genetic differentiation in Alpine plant species (Till-Bottraud & Gaudeul 2002). However, our knowledge of the adaptive potential in plants from the Alps (e.g. Hautier *et al.* 2009) as well as on the adaptive plasticity in Alpine plants (e.g. Pluess & Stöcklin 2005) is very limited. In addition to recent environmental variation, adaptive differentiation may be related to glacial history (Lagercrantz & Ryman 1990). In the Alps, adaptation to historical conditions is likely, since the glacial refugia probably comprised a broad range of different environments to which species needed to adapt in order to survive (Hewitt 1996).

Distribution in Alpine plants

One of the most basic questions in biogeography is what factors affect the distribution of plant species. It was already asked by Darwin in the 19th century (Stöcklin & Höxtermann 2009). On the other side, the spatial structure of plant populations within a species and gene flow dynamics within landscape mosaics are central issues in landscape ecology (Silvertown & Antonovics 2001). From the point of view of metapopulation theory, distributions of plant populations may be explained by frequent extinctions and colonisations of suitable habitats. There is clear evidence that plant populations are temporally and spatially structured and are interconnected through gene flow by seeds or pollen (Ouborg & Eriksson 2004), although the relevance of such metapopulation processes for plant distribution has been questioned (Freckleton & Watkinson 2003).

For distribution of plant species at the regional scale, seed dispersal is one crucial aspect (Münzbergova & Herben 2005). In the highly structured landscape of the Alps, long-distance dispersal might often be the only way for seeds to reach new habitats located far away from the source population (Bacles *et al.* 2006). Therefore, Alpine plants without any morphological adaptations for seed dispersal may be dispersal limited, since they are disadvantaged in colonisation of new habitats (Stöcklin & Bäumler 1996). Another important factor for regional distribution of plants is the availability of suitable habitats (Münzbergova & Herben 2005). Therefore, naturally or anthropogenically caused large-scale disturbances may lead to landscape fragmentation and thereby a loss of habitats for Alpine plant species.

At the local scale, a species' distribution is theoretically fully limited by the availability of either seeds or microsites. However, most often in nature, the local distribution is limited by a complex interaction of these two factors (García-Camacho *et al.* 2010). In Alpine plant populations, the availability of seeds may mainly depend on pre-dispersal seed predation and population size (Weppler & Stöcklin 2006). Small-scale disturbances of the soil structure in alpine ecosystems, for example by burrowing mammals, may create ideal microsites for successful germination (Chambers 1995).

Conservation aspects in Alpine plants

The overall plant species richness of vascular plants in the European Alps is surprisingly high, with approximately 4,500 species (Aeschimann *et al.* 2004). Although, diversity of Alpine plant communities and species is currently threatened by environmental changes such as socio-economically motivated changes in traditional land use (Körner 2002). These changes include overgrazing and abandonment of former grassland (Rudmann-Maurer *et al.* 2008). Additionally, global temperature rise has led to species range shifts towards the summits of mountains, which may also decrease the Alpine plant diversity (Parmesan & Yohe 2003; Walther 2010).

Within a plant species, small populations may get extinct, when environmental change leads to habitat loss, because any decrease in population size may immediately reduce viability due to inbreeding and low genetic diversity (‘extinction vortex’; Till-Bottraud & Gaudeul 2002). Therefore, the loss of traditional land use types in the Alps may not only affect whole communities and rare species negatively, but also rendering many plant populations vulnerable to extinction, even within common species (Rudmann-Maurer *et al.* 2007).

There are several reasons why Alpine plant species are worth to be protected. One important reason is surely that the belowground plant structures of a diverse vegetation are better suited to assure the stability of mountain slopes than a species poor vegetation (‘insurance hypothesis’; Körner 2002). Consequently, stable mountain slopes are important for erosion control and for a sustainable Alpine ecosystem that contributes to the benefits of humans living downslope, with water supply to mention one important function (Messerli & Yves 1997).

Overview

Chapter 2 E.S. Frei, J.F. Scheepens, G.F.J. Armbruster & J. Stöcklin
Phenotypic differentiation in a common garden reflects the phylogeography of a widespread Alpine plant
Journal of Ecology, in press

This chapter focuses on the genetic diversity in molecular markers (RAPDs) and phenotypic traits in populations of *Geum reptans* sampled from the European Alps. By combining a molecular study with a common garden experiment, we addressed the important question whether the phenotypic differentiation reflects the phylogeographic structure. We further asked whether phenotypic differentiation is caused by adaptation and we investigated regional differences in competitiveness of plants by using a competition treatment in the common garden. Results were interpreted in the context of Quaternary glaciations and climatic oscillations as well as of more recent environmental variation.

Chapter 3 J.F. Scheepens, E.S. Frei & J. Stöcklin

Glacial history explains regional differentiation in phenotypic traits in an Alpine plant

New Phytologist, submitted

Similarly as in **Chapter 2**, we hypothesised in this study that glacial history in the Alps has affected phenotypic differentiation in *Campanula thyrsoides*. A classical common garden experiment was used to test whether differentiation in morphology and phenology is due to phylogeographic origin, in order to study the impact of isolation in different glacial refugia and recolonisation on phenotypic population differentiation. By including a clipping treatment in the common garden, we additionally investigated regional variation of plants in response to grazing.

Chapter 4 E.S. Frei, J.F. Scheepens & J. Stöcklin

High genetic differentiation and founder effects in populations of a rare Alpine plant on a small mountain plateau

American Journal of Botany, in revision

In this project, we analysed genetic diversity within and among all naturally occuring populations of *Campanula thyrsoides* on a small mountain plateau (Schynige Platte) in the Swiss Alps by using microsatellites. In the analysis, we incorporated data on population characteristics from a long-term monitoring study and compared our results with genetic diversity in this species observed at two larger scales in the Alps. For an ecological interpretation of our findings, we used the available, detailed knowledge of the species including its monocarpic life-cycle, breeding system and dispersal.

Chapter 5 J.F. Scheepens, E.S. Frei, G.F.J. Armbruster & J. Stöcklin

Monocarpic perenniality of *Campanula thyrsoides* results in high population differentiation despite high pollen flow

Annals of Botany, in preparation

Here, we focused on the contemporary gene flow in a single population of *Campanula thyrsoides* on a mountain plateau, in contrast to the previous molecular study (**Chapter 4**), in which both historical and contemporary gene flow were addressed. We mapped and genotyped (microsatellites) all available mother plants and their offspring in a population and used a paternity analysis to estimate pollen flow into this population. We also measured pollen flow directly by means of fluorescent pollen experiments in two consecutive years.

Chapter 6 E.S. Frei, J.F. Scheepens & J. Stöcklin
Dispersal and microsite limitation of a rare alpine plant
Plant Ecology, accepted

In this chapter, we tested the limitations of regional and local spread in *Campanula thyrsoides* on a mountain plateau (Schynige Platte) in the Swiss Alps with a classical seed sowing experiment in two consecutive years. We have selected sowing sites based on different habitat suitability for adult *C. thyrsoides* by using species co-occurrence data from previous vegetation relevées in the same study region. By adding seeds in unoccupied sites and in different seed densities, we tested for dispersal and seed limitation. Furthermore, we asked whether habitats of high suitability for adult *C. thyrsoides* are also advantageous for germination and establishment of seedlings. Based on the results, we propose an appropriate conservation strategy for this rare bellflower.

The last chapter of this doctoral thesis (**Chapter 7**) summarises the main results of all research projects and discusses the most important findings. Furthermore, this chapter highlights aspects of our results in the contexts of conservation and preserving Alpine biodiversity. Finally, an outlook identifies further research priorities based on our present work and the chapter closes with general conclusions.

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Chapter 2

Phenotypic differentiation in a common garden reflects the phylogeography of a widespread Alpine plant

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Summary

1. Glacial history has affected the phylogeographic structure of numerous Alpine plant species, but its impact on phenotypic differentiation has been little studied. Therefore, we asked whether phenotypic differentiation in a common garden reflects the phylogeographic structure of the widespread Alpine plant *Geum reptans* L.

2. We combined a molecular investigation with a common garden experiment and investigated genets from 16 populations of *G. reptans* sampled from the European Alps. Using neutral molecular markers (RAPDs) and Bayesian cluster analysis, we analysed the species' genetic differentiation and phylogeographic structure. In the common garden, we measured the differentiation of phenotypic traits related to growth, reproduction and leaf morphology.

3. Molecular analysis partitioned the populations into three genetic groups, indicating pronounced phylogeographic structure. Regional molecular variation was correlated with regional phenotypic differentiation.

4. Quantitative trait differentiation (Q_{ST}) differed from neutral molecular differentiation (G_{ST}) for 10 out of 11 traits, indicating that selection has contributed to phenotypic differentiation. Significant negative correlations between biomass and precipitation records for site of origin are a further indication of adaptation.

5. *Synthesis*. The current study compared regional molecular variation and phenotypic differentiation among populations of a widespread species in the context of extreme range changes during glaciations in the Alps. Because the common garden phenotypic differentiation of *G. reptans* reflects its phylogeographic structure, we conclude that glacial history affected both genotypes and phenotypes. The results suggest that the present-day phenotypic differentiation was caused by genetic drift and limited gene flow between populations in glacial refugia and during postglacial recolonisation, as well as by adaptation to current climatic conditions. Our findings are relevant for understanding the adaptive potential of Alpine plants and predicting potential range shifts in response to future climate change.

Key-words: adaptation, common garden, ecological genetics and ecogenomics, genetic drift, *Geum reptans*, glacial history, phenotypic differentiation, Q_{ST}-F_{ST} analysis

Introduction

Our understanding of the historical background of regional differences within plant species has increased substantially since the development of molecular methods (Sunnucks 2000). In the last decade, particular attention has been paid to the way in which the genetic makeup of species has been impacted by the cyclical and extreme range changes that occurred during climatic oscillations and glaciations in the Quaternary (Hewitt 1996, 2000; Taberlet *et al.* 1998). In the European Alps, phylogeographic studies have demonstrated that the effects of population survival in different glacial refugia outside the Alps are still detectable in regional molecular differentiation in numerous Alpine plant species (Schönswetter *et al.* 2005; Alvarez *et al.* 2009). Genetic drift, and gene flow through pollen and seed dispersal, are considered to be the main opposing evolutionary forces responsible for neutral molecular differentiation in plants (Till-Bottraud & Gaudeul 2002). Therefore, neutral drift and subsequent limited gene flow during glacial survival and recolonisation of the Alps may have led to the observed phylogeographic differentiation between populations of Alpine species (Schönswetter *et al.* 2005). Molecular phylogeographic differentiation is detectable between present-day populations of Alpine plants for two reasons. First, the period of time since the last glaciation (c. 10,000 years) is too short for different phylogeographic lineages to have been obscured by interbreeding. Second, current gene flow is too weak to have completely masked historical effects because it is limited by dispersal barriers, such as the deep valleys and high mountain chains characteristic of the Alps (Körner 2003).

It can be hypothesised that glacial history has also had an impact on the phenotypic differentiation of Alpine species. In contrast to random genetic drift, which leads to neutral differentiation (Nei *et al.* 1975), selection leads to adaptive differentiation that maintains or enhances fitness in different environments (Kawecki & Ebert 2004). Adaptive regional differentiation of phenotypic traits has been well documented in widespread plant species (Joshi *et al.* 2001; Olsson & Ågren 2002; Becker *et al.* 2006) and can be expected in Alpine species, firstly because Alpine plants may have experienced historical selection caused by local conditions in glacial refugia outside the Alps (Hewitt 1996), and second, because selection caused by current environmental conditions may result from both regional climatic differences over the Alpine belt and local spatial and temporal heterogeneity of distinct Alpine habitats (Till-Bottraud & Gaudeul 2002). Therefore, both neutral processes occurring during glaciations and postglacial recolonisation, and historical and current adaptive processes may have affected phenotypic differentiation in widespread Alpine species.

Whereas selection leading to adaptation is a long-term process requiring many generations, phenotypic plasticity allows more rapid adjustment to environmental variation at a more fine-grained scale (Sultan 2000). Phenotypic plasticity complements phenotypic differentiation and is considered a genetic trait in itself (Schlichting & Smith 2002). Regional variation in adaptive plasticity has been observed in lowland species (e.g. Berg *et al.* 2005) and might be particularly pronounced in Alpine species because their habitats are subject to wide environmental variation (Gonzalo-Turpin & Hazard 2009).

Most of the phylogeographic studies that have investigated the impact of glacial history on within-species differentiation have used only neutral molecular markers (for a review, see Schönswetter *et al.* 2005). The present study is among the first to directly compare molecular differentiation to phenotypic differentiation in the phylogeographic context of Alpine glaciations (but see Lagercrantz & Ryman 1990). Here, we investigated whether the phylogeographic structure inferred from putatively neutral molecular markers (RAPDs) is reflected in differentiation of phenotypic traits related to growth, reproduction and leaf morphology. We analysed genets of the widespread Alpine species *Geum reptans*. Genets were sampled from 16 populations across the species' range in the Alps (Fig. 1) and used in a common garden experiment, as well as for molecular analysis. Common garden experiments are a powerful tool for revealing genetic differentiation in the phenotypic traits of plants from different regions and populations (e.g. Olsson & Ågren 2002). When a treatment such as competition is included in a common garden experiment, phenotypic plasticity can be measured as variation in the response to this treatment (Pluess & Stöcklin 2005). Therefore, we grew clonal progeny of *G. reptans* in a common garden with and without the alpine grass *Poa alpina* L., to test for genetic differentiation in competitiveness. Common garden experiments are not suitable for distinguishing between neutral evolutionary forces (such as drift) and adaptive processes as the causes of phenotypic variation (Kawecki & Ebert 2004). Nevertheless, correlations between traits measured in a common garden, and environmental variables at the original sites of the populations may suggest adaptation (Linhart & Grant 1996), and comparisons of Q_{ST} and F_{ST} may indicate whether phenotypic trait differentiation is affected by selection (Merilä & Crnokrak 2001). Neutral molecular differentiation (F_{ST}) is a measure of background genetic drift, and any deviation in quantitative trait differentiation (Q_{ST}) from F_{ST} indicates selection (Spitze 1993).

We addressed the following questions in our study. (1) Is the regional phylogeographic structure of *G. reptans* inferred from putatively neutral molecular markers correlated with common garden phenotypic differentiation? (2) If present, is the regional differentiation in molecular markers and phenotypic traits more pronounced than population differentiation within regions? (3) Are there indications that, in addition to neutral genetic drift, adaptive processes have affected phenotypic differentiation? (4) Is there regional variation in competitiveness?

Materials and methods

Study species

The distribution of *G. reptans* encompasses the entire European Alps extending eastward to the Carpathians and the mountains of northern Albania and Bulgaria (Conert *et al.* 1995). The species occurs predominantly on moraines in glacier forelands, moist screes and mountain ridges of siliceous bedrock (Aeschimann *et al.* 2004). *Geum reptans* is one of the first pioneers on virgin soils after glacier retreat and persists until competition with other species becomes too strong (Weppler & Stöcklin 2005). Plants reproduce sexually, by producing 1–5 flowering stems with terminal flower heads, and clonally, by forming new rosettes at the tip of stolons (Pluess & Stöcklin 2005).

Common garden experiment

In the late summer of 2007, plant material was collected from 16 populations of *G. reptans* at different sites (Fig. 1; see also Appendix 1). To obtain representative coverage, we collected samples from an area that spanned all biogeographic regions that are assumed to reflect spatial genetic structure within Alpine plant species (Schönswetter *et al.* 2005). From each population, we sampled a minimum of four stolons with rosettes (ramets) from 20 genets. Each genet was at least 5 meters from others to minimise the risk of resampling genotypes. Rosettes were kept in plastic bags in a refrigerator for 5–12 days until they were planted in separate 10 x 10 x 10 cm^3 pots filled with a 1:1 mixture of river gravel and potting soil. Pots were placed on tables in a greenhouse and their distribution on the tables was randomised weekly. We applied the organic insecticide Traunem (Andermatt Biocontrol AG, Grossdietwil, Switzerland) to the plants twice to control infestations of Sciaridae. Four weeks before transplantation, the plants were placed outdoors for acclimatisation.

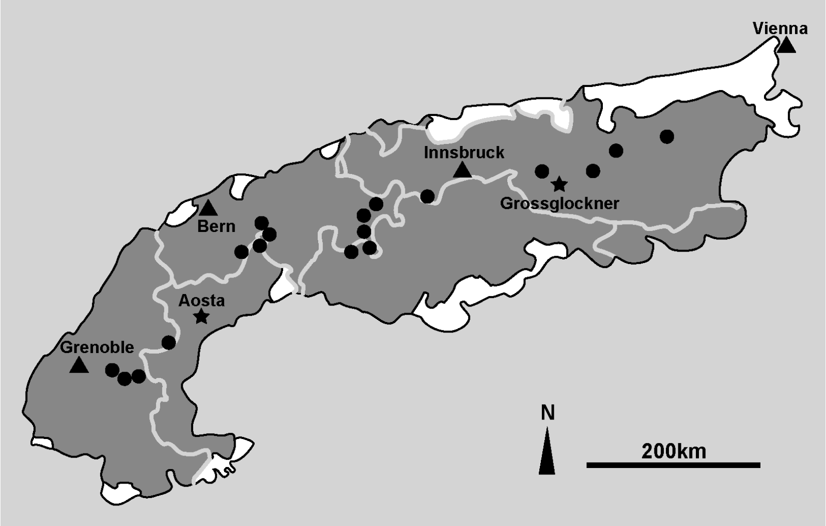

Fig. 1. Locations (dots) of populations of *Geum reptans* sampled from the European Alps. Dark grey areas show where the species occurs and white areas where it is absent. Light grey lines represent the borders of the Alpine countries. Map modified from Aeschimann *et al.* (2004).

On 19 May 2008, the plants were transferred to a common garden in the Central Alps in Davos (altitude 1,532 m). From each of the 16 populations, we planted four ramets of 8–14 genets each in the garden ($n = 592$ plants). We used a randomised block design with each sampled population represented by an equal number of individuals in each of four blocks. Two of the four ramets of each genet were surrounded by seedlings of *Poa alpina* (seeds from the Austrian Alps, Otto Hauenstein Samen, Landquart, Switzerland) to provide interspecific competition and to enable us to measure competitiveness. The plants did not require watering, but the garden was weeded regularly, and *Poa alpina* was clipped four times to prevent it becoming too competitive.

Initial plant diameter was measured immediately after transplantation. Traits related to growth (number of leaves), reproduction (numbers of reproducing individuals, flowers and stolons) and morphology (length, width and number of leaflets of the longest rosette leaf, and specific leaf area) were measured after two growing seasons in June 2009. On 3 July 2009, plants were harvested, and leaf and root biomass were measured separately after drying at 80°C for 72 h. For biomass partitioning, we calculated root mass as a percentage of total biomass (sum of leaf and root mass) for each plant. To quantify competitiveness, we followed Snaydon (1991). First, for each genet, we calculated the difference in the average *log* (biomass) between ramets from plants grown with and without competition. Second, to obtain a relative measure of competitive ability, we subtracted the previously calculated difference from one. Higher relative competitive ability of a genet indicated stronger phenotypic plasticity. As a measure of the relative importance of clonal versus sexual reproduction, we calculated the clonality, i.e. the proportion of stolons on all reproductive meristems (flowers and stolons). As an indicator of leaf shape, we calculated the ratio of leaf length to leaf width. To estimate the number of leaflets, we counted all secondary veins branching from the leaf midrib, and as a measure of leaf dissection, the number of leaflets was divided by leaf length. Specific leaf area (SLA) was measured in a subset of plants without competitors ($n = 125$). Five circular leaf corings with an area of 44 mm^2 each were taken from different rosette leaves of an individual plant and dried at 60°C for 48 h. All leaf corings from an individual plant were weighed together. SLA was then calculated as the fresh leaf area divided by the dry weight of the corings (Cornelissen *et al.* 2003).

RAPD fingerprinting

Leaf material from eight genets from each population in the common garden experiment ($n = 128$) was analysed using RAPD fingerprinting (Williams *et al.* 1990). DNA extraction from dried leaf material and measurement of the DNA concentration were performed as described in Pluess and Stöcklin (2004). After a pilot study to search for suitable primers, we selected the following five oligos for fingerprinting: X5[CGGTCACTGT], M6[GTGGGCTGAC], OPP17[TGACCCG CCT], OPP8[ACATCGCCCA] and OPP9[GTG GTCCGCA]. RAPD-PCR was performed using self-dissolving Illustra^{TM} puReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK). The beads contained 10 mM Tris-HCl buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl and 2.5 U. In addition, 6 ng of DNA, 25 pmol primers and ddH_2O to a final volume of 25 μL were added to each PCR bead. PCR amplifications were always run in the same machine (Mastercycler gradient, Eppendorf, Hamburg, Germany) with the following conditions: 120 s at 94°C for initial denaturing, followed by 34 cycles of 92°C for 30 s, 36°C for 30 s and 72°C for 90 s, with a final extension step of 72°C for 300 s. PCR products were separated on 2% agarose gels in 1x tris-borate-EDTA buffer with 100-bp DNA ladders as size standards. Gels were stained with ethidium bromide.

We scored only clear and distinct bands and tested the repeatability of the banding pattern (the absence or presence of bands) in 15 genets with a second complete RAPD analysis (Weising *et al.* 2005), which revealed an error rate of 4.6%. For data analysis, both monomorphic and polymorphic bands were taken into account (Nei 1973).

Molecular analyses

To estimate the genetic diversity within populations, we calculated the expected heterozygosity (H_e; Nei 1973) for each population using POPGENE version 1.3 (Yeh *et al.* 1997). G_{ST}, a measure of the genetic differentiation between populations (Nei 1973), was estimated using the same program, and 95% confidence intervals were obtained through jackknifing over populations (Miller 1974).

To investigate the genetic structure of the populations, we used a model-based Bayesian cluster analysis to assign genets to genetic clusters. We used the algorithm for dominant markers (Falush *et al.* 2007) and a standard admixture model with independent allele frequencies (Pritchard *et al.* 2000) in the program STRUCTURE version 2.3. After a burn-in period of 100,000 cycles, 100,000 Markov Chain Monte Carlo simulations were performed for values of K (the number of clusters) ranging from 1–10. The *ad hoc* statistic ΔK was used to identify the most likely number of clusters within the dataset (Evanno *et al.* 2005). Molecular data from four genets from each of two additional populations (STAU and TTAU, see Appendix 1) from the Eastern Alps were included in the cluster analysis to check the continuity of the easternmost phylogenetic group (Fig. 2). These two additional populations were not included in the common garden experiment. Leaf material for these populations was provided by the IntraBioDiv Consortium (Gugerli *et al.* 2008).

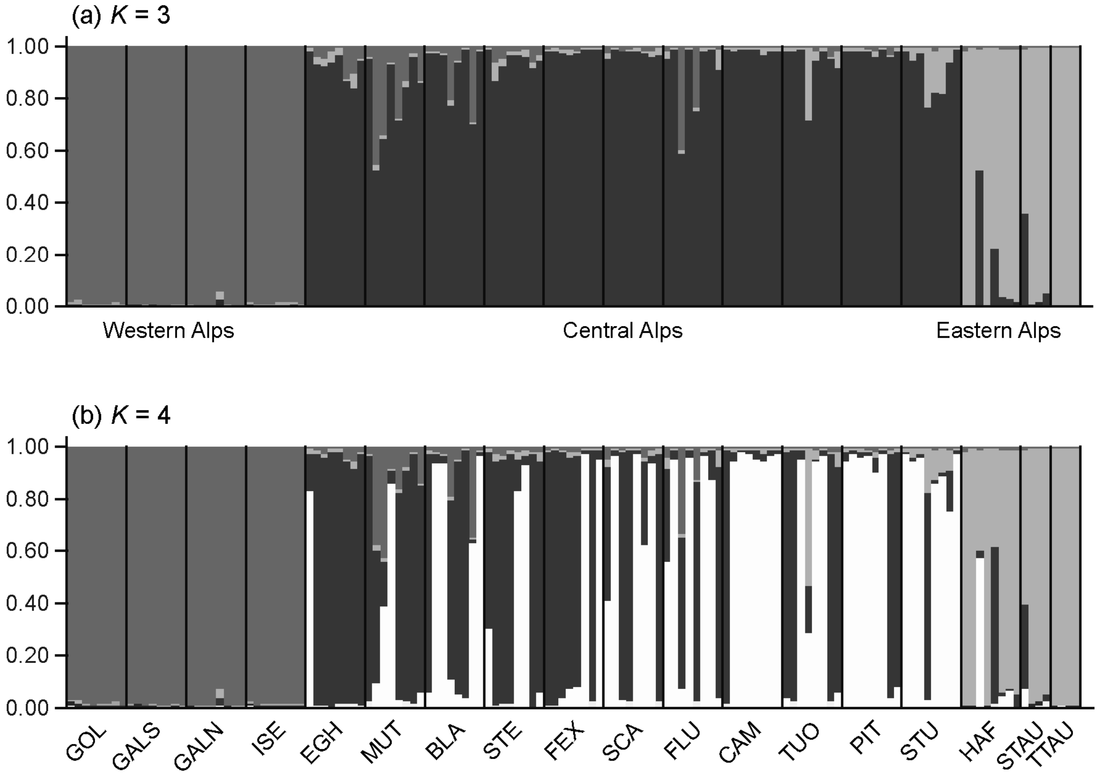

Fig. 2. Molecular differentiation of genets from populations of *Geum reptans* sampled from the Alps for (a) $K = 3$ clusters and for (b) $K = 4$ clusters inferred from Bayesian cluster analysis using the program STRUCTURE. The different clusters (phylogeographic regions) are represented by different shades of grey. Genets are grouped to populations which are aligned from the Western (left) to the Eastern Alps (right). Bars indicate the assignment probability (*Q*) of genets to be a member of one of the clusters. The graph shows the simulation run with the highest likelihood for the posterior distribution (*Ln P*) of data out of 20 runs for each *K*.

To test for isolation by distance (Wright 1946), we correlated pairwise genetic distances (Nei 1978) with the geographic distances between populations using Mantel tests. We did this for populations from all phylogeographic regions, and for populations from the Central Alpine region separately in GENALEX version 6.0 (Peakall & Smouse 2006). Assignment of populations to regions is described in detail below. The significance level of the Mantel correlation coefficient *R* was obtained after performing 1,000 permutations. The partitioning of molecular variance between regions, populations within regions and genets within populations was determined using an AMOVA (Excoffier *et al.* 1992). Fixation indices were computed and tested using 1,000 permutations for each level of the genetic structure: Φ_{RT} for variation between regions, Φ_{PR} for variation between populations within regions and Φ_{PT} for variation within populations. AMOVA and fixation indices were computed using GENALEX.

Prior to statistical analysis of the common garden experiment, we assigned the populations to the phylogeographic regions inferred from the Bayesian cluster analysis of molecular data. These regions included the Western Alps, Central Alps and Eastern Alps (Fig. 2a). Each population was assigned to a region when its probability of assignment (Q) to one of the three clusters ($K = 3$) was higher than 70% in the simulation run that had the highest likelihood for the posterior distribution ($Ln\ P$) of data out of 20 runs. We used three regions because for four regions ($K = 4$; Fig. 2b), the assignment probabilities were too weak to clearly assign the populations from the Central Alps into two groups. We also did not find a split into two well-separated groups when the Bayesian analysis using STRUCTURE was repeated for only the Central Alpine populations (results not shown).

Linear modelling

We used mixed-effects modelling to investigate genetic effects (effects of phylogeographic region, population and genet) and environmental effects (effects of competition) on traits measured in the common garden. To analyse the frequency of reproduction with a binomial error distribution, we fitted generalised linear mixed models (GLMMs) with a *logit* link function. For all continuous variables with normal error distributions, we fitted linear mixed models (LMMs). In both models, we used restricted maximum likelihood (REML). These models perform better with unbalanced datasets (in this case, an unequal number of populations per region) than classical ANOVAs (McCulloch & Searle 2001). Mixed-effects models were calculated using the function *lmer* in the R package LME4 (Bates & Maechler 2009). The most complex model included the initial plant diameter as a covariate and the factors *Competition* and *Region*, as well as their interaction, as fixed effects. The factors *Block*, *Population* (nested in *Region*) and *Genet* (nested in *Population*) were treated as random effects in the model. The covariate was included to account for initial size differences. *Block* was included as a random effect to account for possible spatial heterogeneity in the common garden. To test for the significance of the fixed effects, conditional F-tests were performed as recommended for mixed-effects models (Faraway 2006). We estimated random effects by calculating their variances and tested the significance of the random effects using likelihood ratio tests following Pinheiro and Bates (2000). We checked all model assumptions using diagnostic plots constructed in the R packages LATTICE (Sarkar 2009) and ASUR (Fabbro 2007). Biomass, number of leaves, number of flowers and stolons were natural logarithm-transformed to conform with model assumptions. Tukey's HSD post-hoc tests were used to test differences between means of trait values for each pair of regions.

To determine how much variation in experimental plants' traits could be attributed to genetic effects, we used linear models with the factors *Region*, *Population* and *Genet* nested in each other and fitted as random effects. The variances were extracted from the models with the function *VarCorr* in the R package LME4 (Bates & Maechler 2009), and the corresponding variance components (V) were calculated based on Crawley (2007).

To analyse regional variation in phenotypic plasticity, we fitted linear models with the relative competitive ability in terms of growth (i.e. of leaf and root mass). The effects of *Region*, *Population* and *Genet* were nested in each other and tested with ANOVAs. Another ANOVA was run to test for regional differences in genetic diversity (H_e). Contrast tests using the function *mancontr* in the R package ASUR (Fabbro 2007) were performed to examine differences between means of the competitive abilities between regions and between means of the H_e values between regions.

All statistical analyses described above were performed using the statistical language R version 2.10.0 (R Development Core Team 2009).

Q_{ST}-F_{ST} analysis

To evaluate whether any of the observed phenotypic differentiation was due to selection, we compared the quantitative trait differentiation (Q_{ST}) of all phenotypic traits measured in the common garden with a neutral molecular differentiation index (G_{ST}). In theory, when a trait is differentiated in a neutral manner, Q_{ST} should equal G_{ST}. In contrast, a trait is assumed to have been under selection when Q_{ST} differs from G_{ST}; i.e. unifying selection has occurred when $Q_{ST} < G_{ST}$, and diversifying selection has occurred when $Q_{ST} > G_{ST}$ (Merilä & Crnokrak 2001). We calculated Q_{ST} according to the formula used by Spitze (1993). Instead of extracting variance components from classical ANOVAs (Spitze 1993), we used a REML approach and calculated mean Q_{ST} values and 95% confidence intervals through jackknifing over populations (O'Hara & Merilä 2005). To investigate whether Q_{ST} differed significantly from G_{ST}, we checked whether the 95% confidence intervals of the mean Q_{ST} values overlapped with the G_{ST} value. All calculations were performed using R version 2.10.0 (R Development Core Team 2009).

Correlation analysis

To determine whether observed trait differentiation was related to climate, we performed a Pearson's correlation analysis in R version 2.10.0 (R Development Core Team 2009) between all phenotypic traits and climatic data from the site of population origin. Climatic data were obtained from the WorldClim database (http://www.worldclim.org), a set of global climate grids with a spatial resolution of 150 arc-seconds containing monthly climatic data from 1950–2000 (Hijmans *et al.* 2005). From the WorldClim data points surrounding each site, we selected the one that differed least in altitude from the population location. Temperature data were corrected for the difference in altitude by adding or subtracting 0.0055°C m^{-1} (Ozenda 1988). We calculated total annual precipitation, annual summer temperature (mean for the months June–August), and mean, minimum and maximum annual temperatures based on the monthly climatic data. We averaged the annual data for each parameter over the last fifty years. Because variables other than temperature can change with elevation, we analysed correlations between the altitude of the site of origin and all phenotypic traits. By correlating climatic data and the altitude of the site of origin with the residuals obtained using ANOVAs on phenotypic traits with *Region* as fixed effect, we could make a stronger case for adaptation. This is because the region effect could be due to both neutral differentiation and regional adaptation, whereas correlations after the removal of the region effect would indicate Alpine-wide adaptation to local conditions.

Results

Molecular differentiation and phylogeographic structure

A total of 53 different RAPD markers were scored in all investigated genets. Only two of these 53 markers were monomorphic. The genetic diversity in all the studied populations of *G. reptans* was $H_e = 0.14 \pm 0.04$ (mean ± SD), with a range of 0.07–0.21. The values of H_e differed significantly between phylogeographic regions (ANOVA (*Region*): $F_{2,15} = 11.89$, $P < 0.001$), with the lowest genetic diversity $H_e = 0.08 \pm 0.01$ (mean ± SD) occurring in the West Alpine populations (Fig. 3a). The average genetic differentiation between populations was $G_{ST} = 0.395$ (95% CI 0.388–0.399).

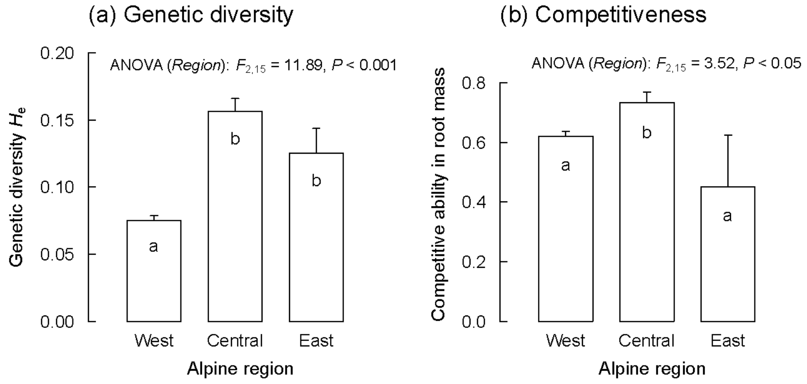

Fig. 3. Regional differences in (a) genetic diversity and (b) competitive ability in root mass of genets from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps. Genets were grown with and without competition from the grass *Poa alpina* in a common garden to measure competitiveness (sensu Snaydon 1991). Bars show means + SE. *F*- and *P*-values are from ANOVAs. Significance of differences between regions was obtained with contrast tests at the $\alpha = 0.05$ level and indicated by different letters.

Bayesian cluster analysis of the molecular data resulted in a distinct phylogeographic structure, with three genetic clusters ($K = 3$) having the best *ad hoc* statistical fit (ΔK; Appendix 2). For $K = 3$, the populations were grouped into West, Central and East Alpine groups (Fig. 2a). For $K = 4$, the Central Alpine group was split into two groups, dividing the Central Alpine populations into western and eastern groups, but with a large admixture zone and no clear geographic boundary (Fig. 2b). Pairwise genetic distances ranged from 0.02–0.27 and were significantly correlated with geographic distances, both when all populations were included in the Mantel test ($R = 0.80$, $P < 0.01$) and when the Central Alpine populations were analysed separately ($R = 0.58$, $P < 0.01$; Fig. 4).

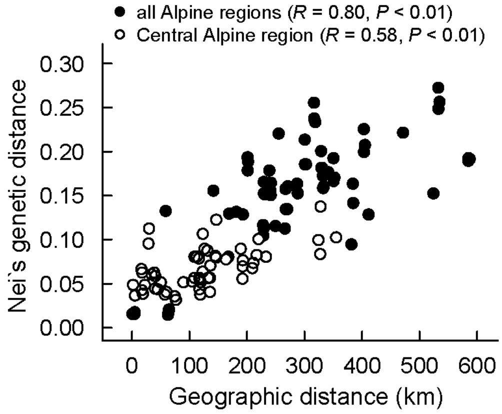

Fig. 4. Correlation of pairwise genetic with geographic distances between 16 populations of *Geum reptans* from three phylogeographic regions in the Alps. The graph shows correlation coefficients and *P*-values from Mantel tests for all populations (filled and open dots) and for Central Alpine populations separately (open dots).

AMOVA revealed that the molecular differentiation between the three phylogeographic regions explained 36% of the total molecular variation, whereas differentiation between populations within regions accounted for only 10% (Table 1).

Table 1. Analysis of molecular variance (AMOVA) with data of genets from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps

Source of variation	df	MS	Estimated variance	Variation (%)	Fixation indices
Between regions	2	86.5	2.6	36	$\Phi_{RT} = 0.36$**
Between populations within regions	13	9.9	0.8	10	$\Phi_{PR} = 0.16$**
Between genets within populations	112	3.9	3.9	54	$\Phi_{PT} = 0.46$**
Total	127	100.3	7.3		

df, degrees of freedom; MS, Mean Squares. Significance of 1,000 permutations: **$P < 0.01$.

Phenotypic differentiation

Significant regional differentiation was present in all the measured traits related to growth, reproduction and leaf morphology (Table 2).

Table 2. Summary of linear mixed model analysis of genetic effects (region, population and genet) and environmental effects (competition) on growth, reproduction and leaf morphology of genets from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps

	Covariate		Competition		Region		Competition x Region		Population		Genet	
	MS	F_1	MS	F_1	MS	F_2	MS	F_2	s^2	χ^2_1	s^2	χ^2_1
Growth												
Total biomass	20.0	58.1***	112	323.8***	1.4	3.9*	0.2	0.5	0.04	10.0**	0.06	6.0*
Leaf mass	21.5	56.5***	152	399.1***	1.4	3.6*	0.2	0.4	0.05	12.8***	0.05	4.9*
Root mass	17.1	51.5***	58.2	175.6***	1.9	5.7**	0.3	0.9	0.03	8.0**	0.08	10.5**
Root mass/total biomass	86.3	2.8	11007	356.2***	279	9.0***	21.0	0.7	8.96	42.0***	8.73	15.7***
No. of leaves	8.2	34.9***	31.5	134.1***	2.1	8.7***	0.1	0.4	0.02	4.8*	0.06	12.8***
Reproduction												
No. of flowers + stolons	0.7	2.6	2.3	8.4**	1. 7	6.3**	0.4	1.6	0.02	2.8	0.11	14.5***
Clonality	1353	2.0	32.5	0.1	669	9.9***	229	0.3	0.01	0.9	6.66	33.9***
Freq. of reproduction	630	24.8***	619	7.0**	591	5.2*	8.0	3.0	0.31	8.7**	2.29	33.1***
Leaf morphology												
Leaflets/length	4.1	16.6***	7.7	31.3***	5.7	23.3***	0.2	0.9	0.03	1.0	0.03	5.0*
Leaf length/width	10.0	2.4	175	72.2***	29.0	12.0***	16.7	6.9**	0.05	1.3	0.32	4.8*
Specific leaf area	2.2	1.4	-	-	14.4	9.1***	-	-	0.07	0.5	0.00	0.0

Fixed effects: Mean Squares (MS) and *F*-values (*F*) are from conditional *F*-tests. Random effects: Variances (s^2) and Chisquare values (χ^2) are from likelihood ratio tests. *Population* is nested in *Region*, *Genet* is nested in *Population*. $n = 462$ for all traits, with exception of $n = 125$ for specific leaf area. The covariate is initial plant diameter. *Block* (random effect) was never significant and is not shown. Significance levels are represented by asterisks: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Plants originating from the West Alpine and East Alpine regions had a greater total biomass, leaf and root mass, and more rosette leaves than Central Alpine plants (Table 3; Fig. 5a). The biomass allocation to roots was highest in the West Alpine plants and decreased towards the east (Fig. 5b). In contrast, the Central Alpine plants had more flowers and stolons than plants from the other two regions (Fig. 5c).

The frequency of plants reproducing either by flowers, stolons, or with both reproductive modes increased (Table 3) as the proportion of stolons on all reproductive meristems (Fig. 5d) decreased from the western to the eastern region. The ratio of leaf length to leaf width and the degree of leaf dissection (number of leaflets divided by leaf length) both increased from the western to the eastern region (Fig. 5e,f). Mean SLA was highest in the West Alpine plants (Table 3).

Table 3. Means (SE) for traits related to growth, reproduction and leaf morphology of genets from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps. Genets were grown with and without competition from the grass *Poa alpina* in a common garden

	Alpine region			Competition	
	West	Central	East	Without	With
Growth					
Total biomass (g)	9.29^{a} (0.6)	6.42^{b} (0.3)	11.13^{a} (2.2)	10.96 (0.5)	3.77 (0.2)
Leaf mass (g)	5.80^{a} (0.4)	4.38^{b} (0.3)	8.10^{a} (1.6)	7.59 (0.3)	2.22 (0.1)
Root mass (g)	3.49^{a} (0.2)	2.04^{b} (0.1)	3.04^{ab} (0.6)	3.37 (0.1)	1.55 (0.1)
Root mass/total biomass (%)	41.1^{a} (0.9)	35.7^{b} (0.5)	27.5^{c} (1.6)	31.2 (0.5)	42.3 (0.6)
No. of leaves	21.4^{a} (0.9)	15.8^{b} (0.6)	32.8^{c} (3.9)	22.9 (0.8)	13.1 (0.6)
Reproduction					
No. of flowers + stolons	1.8^{a} (0.2)	3.0^{b} (0.2)	1.9^{ab} (0.2)	3.0 (0.2)	2.3 (0.1)
Clonality (%)	47.3^{a} (7.0)	21.1^{b} (2.7)	2.6^{b} (0.6)	25.8 (3.4)	23.5 (3.9)
Freq. of reproduction (%)	37.0^{a} (4.7)	48.4^{b} (2.7)	54.2^{ab} (10.4)	50.6 (3.2)	41.4 (3.2)
Leaf morphology					
Leaflets/length (cm^{-1})	1.68^{a} (0.04)	1.90^{b} (0.03)	2.73^{c} (0.13)	1.78 (0.03)	2.02 (0.04)
Leaf length/width	5.04^{a} (0.16)	5.76^{b} (0.11)	7.45^{c} (0.68)	4.99 (0.09)	6.34 (0.16)
Specific leaf area (mm^{2}mg^{-1})	10.48^{a} (0.3)	9.14^{b} (0.1)	9.64^{ab} (0.5)	9.49 (0.1)	-

Mean values identified by the same letter did not differ significantly from one another at the $\alpha = 0.05$ level, using Tukey's HSD post-hoc tests.

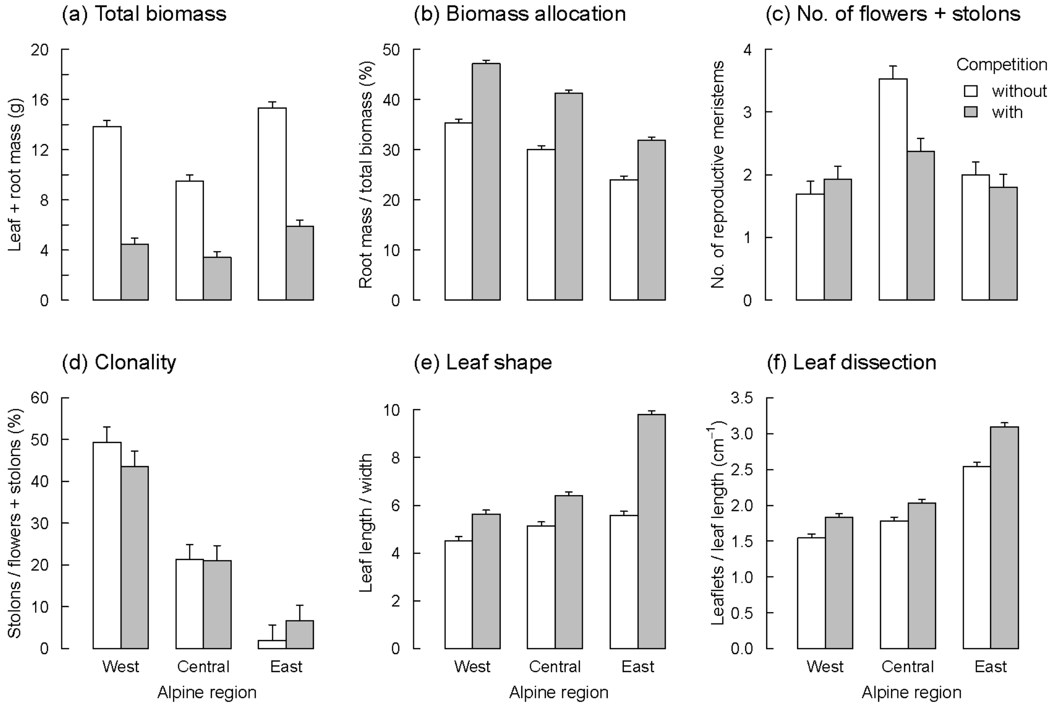

Fig. 5. Quantitative trait differentiation in (a,b) growth, (c,d) reproduction and (e,f) leaf morphology of genets from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps. Genets were grown with and without competition from the grass *Poa alpina* in a common garden. Bars show means + SE based on the pooled error variance from ANOVAs. For significance of differences between regions see letters in Table 3.

Region explained 8.5–27.2% of the variation in growth, 5.7–25.5% of the variation in reproductive traits and 21.6–46.9% of the variation in leaf morphology (Table 4).

Populations within regions were significantly differentiated for six of the 11 traits investigated (Table 2). The initial plant diameter covariate had a significant influence on several traits related primarily to growth (Table 2). *Block* had no significant influence on the investigated traits (results not shown). *Population* and *Genet* generally explained less variation than did *Region*, except in the case of reproductive traits (Table 4).

Table 4. Variance components *V* (%) of genetic effects (region, population and genet) on growth, reproduction and leaf morphology of genets from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps

	Region	Population	Genet
Growth			
Total biomass	10.6	6.5	0.7
Leaf mass	8.5	7.8	0.1
Root mass	15.3	4.6	7.9
Root mass/total biomass	27.2	11.8	0.1
No. of leaves	24.3	3.2	6.9
Reproduction			
No. of flowers + stolons	7.7	4.0	20.9
Clonality	25.5	0.0	34.8
Freq. of reproduction	5.7	2.0	29.4
Leaf morphology			
Leaflets/length	46.9	1.0	3.6
Leaf length/width	26.5	1.3	2.1
Specific leaf area	21.6	2.9	0.0

Differentiation in competitiveness

The competition treatment affected most plant traits significantly. Only clonality was not affected by competition (Table 2). The significant interaction between *Competition* and *Region* on the ratio of leaf length to leaf width (Table 2) indicated that the effect of competition on this trait was different in plants from different regions. The effect of competition was found to be negative for most traits but positive for the biomass allocation to roots, the ratio of leaf length to leaf width and the degree of leaf dissection (Table 3; Fig. 5). Strong regional differentiation in competitive ability was observed for root mass (ANOVA (*Region*): $F_{2,15} = 3.52$, $P < 0.05$). Plants originating from the Central Alps exhibited the highest relative competitive ability for root mass (0.73 ± 0.04, mean ± SE) compared with plants from the other Alpine regions (Fig. 3b). Similar but non-significant trends in competitiveness were observed for leaf mass (results not shown).

Neutral drift versus adaptation

Comparisons of Q_{ST} and G_{ST} indicated that trait differentiation was affected by past selection. Q_{ST} values differed significantly from G_{ST} values, with no overlap between the 95% confidence intervals of Q_{ST} and G_{ST} in any traits, with the exception of the ratio of leaf length to leaf width (Table 5).

Table 5. Estimates of quantitative trait differentiation (Q_{ST}) in growth, reproduction and leaf morphology, and comparisons of Q_{ST} with neutral molecular differentiation (G_{ST}) of genets from 16 populations of *Geum reptans* sampled from the Alps and grown in a common garden

	Q_{ST} (95% CI)	Q_{ST} vs. G_{ST}
Growth		
Total biomass	0.471 (0.450–0.492)	>
Leaf mass	0.610 (0.525–0.695)	>
Root mass	0.421 (0.403–0.440)	>
Root mass/total biomass	0.525 (0.498–0.553)	>
No. of leaves	0.299 (0.278–0.321)	<
Reproduction		
No. of flowers + stolons	0.141 (0.116–0.166)	<
Clonality	0.091 (0.079–0.104)	<
Freq. of reproduction	0.086 (0.077–0.095)	<
Leaf morphology		
Leaflets/length	0.464 (0.421–0.507)	>
Leaf length/width	0.348 (0.311–0.396)	=
Specific leaf area	0.150 (0.140–0.160)	<

CI, confidence interval. Symbols show whether mean Q_{ST} values differed significantly from G_{ST} (mean 0.395; 95% CI 0.388–0.399), indicating diversifying selection (>) or unifying selection (<). Neutral drift (=) is indicated when Q_{ST} was equal to G_{ST}.

Significant correlations of traits with climatic data and the altitude of site of origin were found, suggesting adaptation. Total biomass, leaf mass, root mass and the number of leaves were positively correlated with maximum annual temperature, while all of these traits except root mass were negatively correlated with total annual precipitation (Table 6; Fig. 6a).

Table 6. Correlations of climatic data and the altitude of site of origin with traits related to growth, reproduction and leaf morphology of genets from 16 populations of *Geum reptans* sampled from the Alps and grown in a common garden

	Prec	T_{mean}	T_{min}	T_{max}	T_{summer}	Alt
Growth						
Total biomass	-0.50*	0.46	0.25	0.60*	0.48	-0.21
Leaf mass	-0.55*	0.46	0.26	0.57*	0.47	-0.31
Root mass	-0.33	0.40	0.24	0.57*	0.42	0.03
Root mass/total biomass	0.42	-0.14	0.05	-0.10	-0.15	0.50*
No. of leaves	-0.51*	0.46	0.07	0.69**	0.53*	-0.37
Reproduction						
No. of flowers + stolons	-0.10	0.02	0.14	0.35	-0.22	-0.27
Clonality	-0.09	0.26	0.32	0.29	-0.10	0.24
Freq. of reproduction	-0.43	-0.15	-0.25	-0.11	-0.15	-0.08
Leaf morphology						
Leaflets/length	-0.38	0.14	-0.02	0.12	0.16	-0.49
Leaf length/width	-0.01	-0.14	-0.28	-0.10	-0.13	-0.25
Specific leaf area	0.07	0.44	0.23	0.63**	0.48	-0.05

Values show Pearson's correlation coefficient *r*. Prec, total annual precipitation; T_{mean}, T_{min}, T_{max}, mean, minimum and maximum annual temperatures; T_{summer}, annual summer temperature (mean June–August); Alt, altitude. Climatic data are obtained from the WorldClim database (Hijmans *et al.* 2005) and averaged over the years 1950–2000. Significance levels are represented by asterisks: $*P < 0.05$; $**P < 0.01$.

None of the assessed reproductive traits were correlated with any of the environmental variables (Table 6). Regarding leaf morphology, SLA was positively correlated with maximum annual temperature (Fig. 6b). Finally, the biomass allocation to roots was positively correlated with the altitude of site of origin (Fig. 6c). When the effect of region was removed statistically, the positive correlations of traits with temperature and altitude became insignificant, but the negative correlations of total biomass, leaf mass and the number of leaves with total annual precipitation remained significant (see Appendix 3).

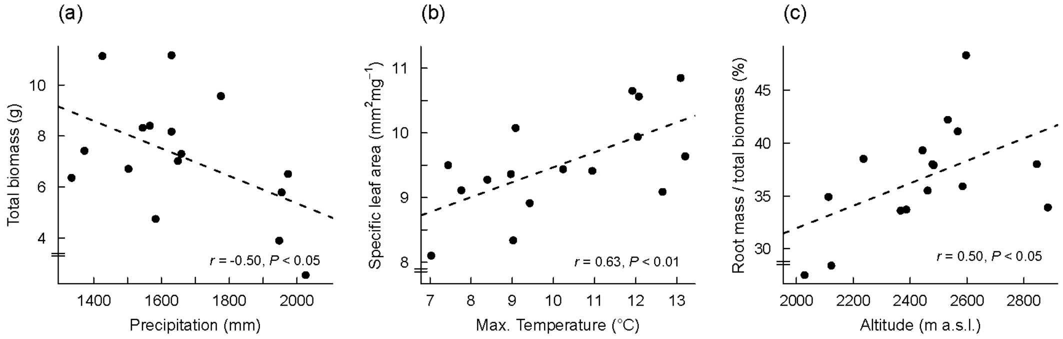

Fig. 6. Correlations of phenotypic traits of *Geum reptans* with (a) total annual precipitation, (b) maximum annual temperature and (c) the altitude of site of origin. Genets from 16 populations of *G. reptans* were sampled from the Alps and grown in a common garden. Climatic data are obtained from the WorldClim database (Hijmans *et al.* 2005) and averaged over the years 1950–2000. The graphs show correlation coefficients and *P*-values from a Pearson's correlation analysis.

Discussion

Molecular differentiation and phylogeographic structure

The average genetic diversity ($H_e = 0.14$) within populations of *G. reptans* was low in comparison with the genetic diversity reported in 20 other widespread plant species ($H_e = 0.22$; Nybom 2004). Genetic bottlenecks resulting from small population sizes (Nei *et al.* 1975) during glacial survival or during postglacial recolonisation might explain this low overall level of genetic diversity. Moreover, genetic diversity was significantly lower in the West Alpine populations than in the populations from the other two regions (Fig. 3a). The lower genetic diversity of West Alpine populations may be caused by their predominantly clonal mode of reproduction (Fig. 5d). The high genetic diversity of the Central Alpine populations (Fig. 3a) may be explained by the ongoing admixture of two previously separated gene pools.

We detected a high level of genetic differentiation between populations of the perennial *G. reptans* ($G_{ST} = 0.40$) compared with the average value inferred for short-lived ($G_{ST} = 0.32$) and long-lived ($G_{ST} = 0.19$) perennials from other RAPD studies (Nybom 2004). The high genetic differentiation observed in *G. reptans* is probably related to its particularly low seed dispersal in the Alpine landscape

(Tackenberg & Stöcklin 2008). In addition, regional differentiation (36%, AMOVA; Table 1) was much higher than population differentiation within regions (10%), which concurs with the distinct phylogeographic structure inferred from Bayesian cluster analysis.

The pronounced molecular phylogeographic structure in *G. reptans* (Fig. 2) fits well with results from previous biogeographic studies of Alpine plants. The two main boundaries that split the Alps into three regions were previously described from floristic data (Merxmüller 1952; Ozenda 1988). A third boundary partitioning the Central Alps into two regions, as previously suggested, e.g. by Ozenda (1988), was not strongly supported by our findings (Fig. 2b). We thus propose that the Central Alpine group may have originated from an admixture of two originally separated gene pools in the central area of the Alps. Two Central Alpine groups were found in a phylogeographic study addressing *G. reptans* that included more populations and used AFLP markers (Thiel-Egenter *et al.* 2009). Our molecular data and the weakly supported previous evidence of a boundary in the Central Alpine region (see Fig. 4b in Thiel-Egenter *et al.* 2009) suggest that gene flow between the two Central Alpine groups is quite substantial, probably because of an absence of pronounced dispersal barriers, despite the isolation by distance pattern (Fig. 4) within the entire Alpine belt. The observed isolation by distance indicates that gene flow is more common between closer populations.

The distinct west–east structure observed in the siliceous species *G. reptans* concurs with glacial refugia on siliceous bedrock being longitudinally oriented at the southern and eastern border of the Alps (Alvarez *et al.* 2009). Therefore, we suggest that the phylogeographic structure and the strong regional differentiation indicated from our molecular analysis is largely a result of genetic drift and limited gene flow during the survival of *G. reptans* in different glacial refugia. Subsequent weak gene flow between Alpine regions due to dispersal barriers, such as the deep valleys in the Western Alps (Aosta valley; Fig. 1) or the high mountain chains in the Eastern Alps (Grossglockner mountains; Fig. 1), may have contributed to the regional differentiation of this species at a later stage.

Phenotypic differentiation

The present study is among the first to compare phenotypic population differentiation to neutral molecular differentiation in the context of glacial history in the Alps. The regional structure of *G. reptans* derived from molecular markers closely parallels the regional phenotypic differentiation observed in this species in the common garden. We detected strong regional differentiation in all the assessed phenotypic traits and for competitiveness (Tables 2 and 3; Fig. 3b). Moreover, the regional phenotypic differentiation was clearly higher than the differentiation between populations within phylogeographic regions (Table 4).

Size differences in plants at the beginning of the experiment influenced several traits significantly (Table 2). The initial size differences might have been a consequence of maternal effects to some degree (Weiner *et al.* 1997). Because we used initial plant diameter as a covariate, these size differences should not have obscured the effects of the tested genetic factors (region, population and genet) and environmental factors (competition).

Several main patterns of regional phenotypic differentiation were found, including i) significantly lower vegetative biomass and more flowers and stolons in Central Alpine plants than in plants originating from the Western or Eastern Alps (Fig. 5a,c), ii) a decrease in the biomass allocation to roots and in clonality from west to east (Fig. 5b,d), and iii) an increase in the ratio of leaf length to leaf width and in the degree of leaf dissection from west to east (Fig. 5e,f). Regional differentiation in competitiveness was also indicated, as the Central Alpine plants suffered less from competition and displayed a higher competitive ability than did plants from the other two regions (Fig. 3b).

We suggest that a home advantage effect could explain the enhanced reproductive output and the high competitive ability of Central Alpine plants because the common garden was located in the Central Alps. Central Alpine plants experienced conditions similar to those existing in their sites of origin, which may have enabled them to achieve higher fitness compared to plants originating elsewhere. However, a home advantage effect cannot adequately explain the decrease of trait values from east to west, or vice versa. Therefore, we conclude that past evolutionary processes, either neutral or adaptive, may have played a role in the regional differentiation of biomass, reproduction and leaf morphology in *G. reptans*.

Neutral drift and glacial history

Our results suggest that strong historical effects, including genetic drift and subsequent limited gene flow during glacial survival and postglacial recolonisation, have affected phenotypic differentiation in *G. reptans*. Differentiation in the ratio of leaf length to leaf width and in the number of leaflets divided by leaf length was especially strong in East Alpine plants compared to those from other regions (Table 3), and this differentiation in leaf morphology might have been caused either by neutral drift in a separate eastern gene pool or by selection. Neutral evolutionary processes leading to differentiation in quantitative traits have frequently been neglected in previous studies of widespread plant species (e.g. Joshi *et al.* 2001; Olsson & Ågren 2002). We emphasise the relevance of such neutral processes for present-day phenotypic differentiation, and suggest that neutral phenotypic differentiation might be a more general phenomenon in widespread Alpine plants than previously assumed.

Indication of adaptation

Some of the observed phenotypic differentiation in *G. reptans* may be explained by historical selection imposed either by environmental conditions during survival in glacial refugia outside the Alps or by current environmental conditions during recolonisation of the Alps. Past selection ($Q_{ST} \neq G_{ST}$) is indicated for almost all traits (Table 5). Thus, selection has played an important role in shaping phenotypic differentiation in this Alpine species, suggesting a relatively high adaptive potential with respect to growth, reproduction and leaf morphology. Strong phenotypic differentiation resulting from adaptation has also been documented in common lowland species (Joshi *et al.* 2001; Becker *et al.* 2006).

To prove that adaptation to particular local conditions has occurred, reciprocal transplantation experiments would be necessary (Kawecki & Ebert 2004). However, adaptation is suggested in our study by the significant correlations of several traits measured in the common garden with climatic data and with the altitude of site of origin (Table 6). For example, biomass and SLA were correlated with climatic variables at the original sites of populations. Reduction in biomass associated with decreasing temperature might be an adaptive strategy of *G. reptans* to reduce freezing damage at locations with low temperatures, as observed in other Alpine plant species (Körner 2003). The reduced biomass in plants from locations with high total annual precipitation (Fig. 6a), which also includes snowfall, could be an adaptation to extended periods of snow cover and a shortened growing season. The negative correlation between biomass and precipitation remained when the regional effect was removed statistically (see Appendix 3), emphasising the importance of precipitation for the local adaptation of traits related to growth. Reduced SLA values (i.e. greater leaf thickness) in plants originating from locations with low temperatures (Fig. 6b) are probably also an adaptation to climatic variation (Scheepens *et al.* 2010). Adaptation to environmental conditions related to altitude is indicated by a significant positive correlation between biomass allocation to roots and the altitude of site of origin (Fig. 6c). An increase in fine root mass with altitude might be related to reduced mycorrhizal infection at high altitudes (Nespiak 1953; Körner & Renhardt 1987). Although comparisons of Q_{ST} and G_{ST} indicated selection for reproduction, none of the reproductive traits were correlated with climatic data or the altitude of site of origin. Therefore, differentiation in reproduction might be partially explained by adaptation to historical conditions or to current environmental conditions that were not measured.

Conclusions

We used a phylogeographic approach to investigate regional phenotypic differentiation in the widespread Alpine plant *G. reptans* at the scale of the European Alps. We demonstrated that the molecular phylogeographic structure paralleled similar, strong common garden phenotypic differentiation. Our results suggest that historical evolutionary forces (such as neutral drift and limited gene flow during survival in glacial refugia and postglacial recolonisation) have affected differentiation of both the genotypes and phenotypes of *G. reptans*. Based on comparisons of Q_{ST} and G_{ST} and correlations of trait values from a common garden with precipitation data from site of origin, we conclude that adaptation to climatic differences may at least partially explain the observed phenotypic differentiation. We suggest that the extreme historical range changes that occurred during climatic oscillations and Alpine glaciations in the Quaternary have left their mark in the phenotypic differentiation patterns of widespread plant species. Therefore, the results of this study might be of relevance for estimating the adaptive potential of Alpine species and the consequences of their potential range shifts in response to future climate change.

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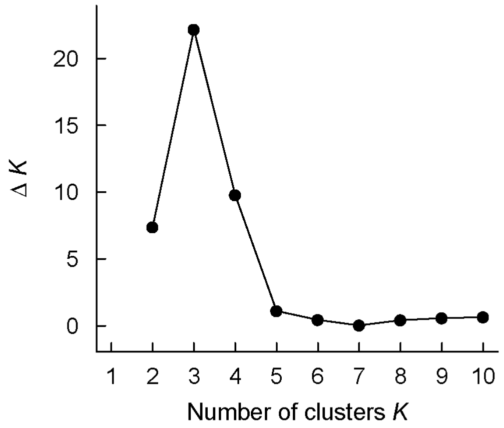
Appendix

Appendix 1. Locations of populations of *Geum reptans* sampled from the European Alps with climatic data and altitude

Alpine region	Location	Population	Lat. °N / Long. °E (WGS84)	Prec (mm)	T_{mean} (°C)	T_{min} (°C)	T_{max} (°C)	Altitude (m a.s.l.)
West	Valfroide, near Lac du Goléon	GOL	45.08200/6.36250	1544	0.4	-10.2	13.1	2443
	South of Col du Galibier	GALS	45.06278/6.40444	1629	-0.3	-10.4	12.1	2567
	North of Col du Galibier	GALN	45.06303/6.40833	1629	-0.4	-10.6	11.9	2596
	Col de l'Iseran	ISE	45.39100/7.04889	1775	0.2	-9.9	12.1	2461
Central	Eggishorn	EGH	46.43000/8.09417	1947	-3.5	-13.4	7.4	2845
	Foreland of Mutt glacier	MUT	46.55750/8.41250	1954	-1.7	-11.4	9.0	2482
	Furkapass, Blauberg	BLA	46.56917/8.41750	2025	-1.9	-11.6	8.4	2532
	Foreland of Steinlimi glacier	STE	46.71167/8.41833	1973	0.3	-9.5	10.9	2112
	Val Fex	FEX	46.36111/9.79528	1582	0.4	-8.6	10.2	2235
	Foreland of Scaletta glacier	SCA	46.70361/9.93722	1565	-0.5	-9.8	9.4	2366
	Flüelapass	FLU	46.74583/9.94556	1502	-1.2	-10.5	9.1	2478
	Vadret da Cambrena	CAM	46.40417/9.99917	1648	-0.5	-9.3	9.0	2386
	Foreland of Tuoi glacier	TUO	46.84000/10.14333	1658	-1.9	-11.0	7.8	2584
	Foreland of Pitztal glacier	PIT	46.92750/10.87806	1372	-3.6	-13.3	7.0	2884
	Stubachtal, Eisboden	STU	47.12528/12.63722	1334	0.1	-11.5	12.7	2122
East	Hafner	HAF	47.08556/13.40417	1425	0.4	-11.0	13.2	2028
	Schladminger Tauern	STAU	47.27508/13.75867	1526	-0.1	-11.4	12.7	2070
	Triebener Tauern	TTAU	47.39409/14.53531	1441	-0.9	-12.8	12.8	2126

Populations are grouped by phylogeographic regions inferred from Bayesian cluster analysis with molecular data. Populations are ordered from the Western (top) to the Eastern Alps (bottom). Climatic data (Prec, total annual precipitation; T_{mean}, T_{min}, T_{max}, mean, minimum and maximum annual temperatures) are obtained from the WorldClim database (Hijmans *et al.* 2005) and averaged over the years 1950–2000.



Appendix 2. *Ad hoc* statistics *ΔK* (Evanno *et al.* 2005) from STRUCTURE analysis with a peak showing the most likely value for $K = 3$ clusters.

Appendix 3. Correlations of climatic data and the altitude of site of origin with phenotypic traits of genets from 16 populations of *Geum reptans* sampled from three different phylogeographic regions in the Alps and grown in a common garden, and with statistical removal of the effect of region

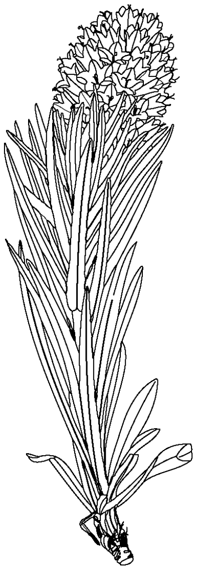
	Prec	T_{mean}	T_{min}	T_{max}	T_{summer}	Alt
Growth						
Total biomass	-0.49*	0.15	0.22	0.04	0.08	-0.10
Leaf mass	-0.50*	0.19	0.22	0.09	0.13	-0.15
Root mass	-0.37	0.01	0.16	-0.09	-0.06	0.06
Root mass/total biomass	0.42	-0.28	-0.10	-0.28	-0.28	0.31
No. of leaves	-0.55*	0.16	0.02	0.20	0.16	-0.17
Reproduction						
No. of flowers + stolons	-0.25	0.35	0.31	0.24	0.28	-0.37
Clonality	-0.21	0.06	0.25	-0.10	-0.03	0.01
Freq. of reproduction	-0.45	-0.06	-0.19	0.04	-0.05	0.02
Leaf morphology						
Leaflets/length	-0.39	0.19	0.21	0.09	0.15	-0.15
Leaf length/width	0.23	-0.19	-0.24	-0.09	-0.20	0.15
Specific leaf area	0.23	0.16	0.11	0.16	0.16	-0.18

The effect of region was removed in the correlation analyses by using the residuals of traits from ANOVAs testing the effect of region on traits (for details see Materials and methods). Values show Pearson's correlation coefficient *r*. Prec, total annual precipitation; T_{mean}, T_{min}, T_{max}, mean, minimum and maximum annual temperatures; T_{summer}, annual summer temperature (mean June–August); Alt, altitude. Climatic data are obtained from the WorldClim database (Hijmans *et al.* 2005) and averaged over the years 1950–2000. Significance levels are represented by asterisks: $*P < 0.05$.

Chapter 3

Glacial history explains regional differentiation in phenotypic traits in an Alpine plant

J.F. Scheepens, **Eva S. Frei** & Jürg Stöcklin



Summary

- Glacial survival in isolated refugia outside the European Alps caused regional differentiation in neutral molecular markers in numerous widespread Alpine plant species. We asked whether glacial history also affected phenotypic differentiation among Alpine regions in plants, using the monocarpic *Campanula thyrsoides* as model species, and whether differentiation is due to adaptation.
- A common garden experiment with 21 populations from across the Alps and Jura Mts. was performed to investigate differentiation among four phylogeographic regions in fitness traits, morphology, phenology and response to clipping.
- Regional differences in phenotypic traits suggest that glacial history is at least partly responsible for regional phenotypic differentiation. Delayed flowering in plants from the Southeastern Alps indicated adaptation to long submediterranean summers, and contrasts with early flowering of plants experiencing short growing seasons in the high Alps to the west. Additionally, the clipping treatment indicated low susceptibility to grazing in plants from the Western Alps, which is in accordance with their predominant occurrence in managed grasslands. Correlations of number of inflorescences, inflorescence height and above-ground biomass with altitude of population origin suggest Alpine-wide adaptation to local environmental conditions.
- This study showed that glacial history can have strong and long-lasting influence on the phenotypic evolution of Alpine plants.

Key-words: adaptation, *Campanula thyrsoides,* common garden, European Alps, genetic drift, glacial history, phenology, phenotypic differentiation

Introduction

Glacial history, i.e. the recurring processes of retreat, glacial survival and recolonisation (Hewitt 1996), has had major consequences for intraspecific evolution of widespread Alpine plants (Schönswetter *et al.* 2005; Thiel-Egenter *et al.* 2011) and likely has led to numerous allopatric speciation events (Hewitt 1996, 2004). Numerous molecular studies have demonstrated that glacial history has left a genetic signature in the spatial genetic structure found within Alpine plant species as a result of long-term survival in isolated refugia on the fringes of the Alps (e.g. Schönswetter *et al.* 2005; Paun *et al.* 2008; Alvarez *et al.* 2009; Thiel-Egenter *et al.* 2009). It can be hypothesised that in widespread Alpine plants glacial history not only caused differentiation in neutral molecular markers, but in a similar pattern also in phenotypic traits through processes of selection and drift (Hewitt 1996).

Phenotypic differentiation among populations of a species can be caused by stochastic processes or selection. Stochastic processes include mutations and neutral genetic drift, increasing differentiation among populations, and gene flow via dispersal of seeds and pollen, which decreases differentiation among populations. Selection is non-random as it favours survival and reproduction of certain phenotypes over others. It is likely that through glacial history both random and selective processes have affected phenotypic traits of widespread plants from the European Alps, for several reasons: 1) for Alpine plants with short and intermediate longevity, the time scale of glacial cycles is long enough for genetic drift and mutations to arise (Klekowski 1997; Schönswetter *et al.* 2005); 2) founder events and bottlenecks are likely during the rapid postglacial recolonisation of the Alps (Hewitt 2004); 3) refugia, mostly located on the fringes of the Alps, probably comprised a variety of environments (e.g. climate, edaphic conditions; Schönswetter *et al.* 2005) to which populations should have adapted in order to survive. Additionally, adaptations to local environmental conditions, especially to factors correlating with altitude (Monty & Mahy, 2009), most likely appeared during postglacial recolonisation.

The monocarpic perennial *Campanula thyrsoides* is genetically subdivided into four major phylogeographic regions arranged longitudinally across the European Alps and Jura Mts. (Kuss *et al.* 2007, 2011; Ægisdóttir *et al.* 2009) as has been found in other species (e.g. Schönswetter *et al.* 2005; Alvarez *et al.* 2009). This arrangement is congruent with major biogeographic distribution patterns based on floristic data (Merxmüller 1954; Ozenda 1988). The phylogeographic regions (Fig. 1) delimited on the basis of molecular markers are roughly located (i) in the Jura Mts. and Western Alps (WA) from Nice to Aosta, (ii) in the Central Swiss Alps (CSA) from Aosta to Lake Como, (iii) in the Central Austrian Alps (CAA) from Lake Como to the Dolomites and (iv) in the Southeastern Alps (SEA) from the Dolomites eastwards (Kuss *et al.* 2007, 2011).

We hypothesise that differentiation in phenotypic traits in *C. thyrsoides* should be observed among phylogeographic regions as a result of glacial history, whether this be through drift or past selection processes. We test this hypothesis by measuring phenotypic traits of plants of 21 populations sampled from across the distribution and grown in a common garden. Common garden experiments allow the quantification of genetic trait differentiation among regions, populations and seed families and are therefore an appropriate method to investigate effects of glacial history on phenotypic differentiation. We included a clipping treatment to simulate grazing or mowing because *C. thyrsoides* frequently occurs in alpine pastures and meadows (Aeschimann *et al.* 2005). Plants from the four phylogeographic regions may respond differently to the treatment, indicating differentiation in phenotypic plasticity in susceptibility to grazing (Pluess & Stöcklin 2004; Suzuki 2008). Finally, we test for postglacial adaptation by calculating correlations between phenotypic traits and altitude of origin across the sampled populations.

Materials and methods

Study species

Campanula thyrsoides L. (Campanulaceae) was used for this study because of its Alpine-wide distribution and occurrence in the adjacent lower mountain ranges of the Dinarids and the Jura Mts. (Aeschimann *et al.* 2005). The plant is a monocarpic bell flower occuring in subalpine and alpine grasslands on calcareous soils or carbonate-rich schists, frequently in moderately disturbed systems, whether natural (steep slopes with unstable soil) or managed (mowing or grazing; Kuss *et al.* 2007). These disturbance regimes positively affect seedling establishment (Frei *et al.* 2011, in press) and may reduce competition with other plants. Two morphologically, geographically and ecologically distinct subspecies have been recognised: subsp. *thyrsoides* in most of the European Alps and Jura Mts. (in phylogeographic regions WA, CSA, CAA), and the subsp. *carniolica* in the Dinarids (Scheepens *et al.* 2011; in SEA). The species' altitudinal distribution typically ranges from 1,600 to 2,200 m a.s.l. (Kuss *et al.* 2007), but reaches lower altitudes in the Jura Mts. and Dinarids, with the lowest population recorded at 217 m a.s.l. near Gračnica, Slovenia (Jürg Stöcklin, pers. obs.). The species is characterised by isolated populations of several hundred to a few thousand individuals (Kuss *et al.* 2008). Initiation of flowering is rosette-size dependent, and Kuss and co-workers (2008) estimated the average flowering age at about 10 years using integral projection models and herb chronology. However, flowering age is highly variable (range 3–16 years; Kuss *et al.* 2007), and under benign conditions in a common garden the large majority of plants flowers in the second year (Scheepens *et al.* 2010). The outcrossing species has a strong but incomplete self-incompatibility system (Ægisdóttir *et al.* 2007).

Common garden experiment

Six seed families each from 21 populations were sampled in four phylogeographic regions across the Alps and the Jura Mts. (WA, CSA, CAA, SEA; Fig. 1, Appendix 1).

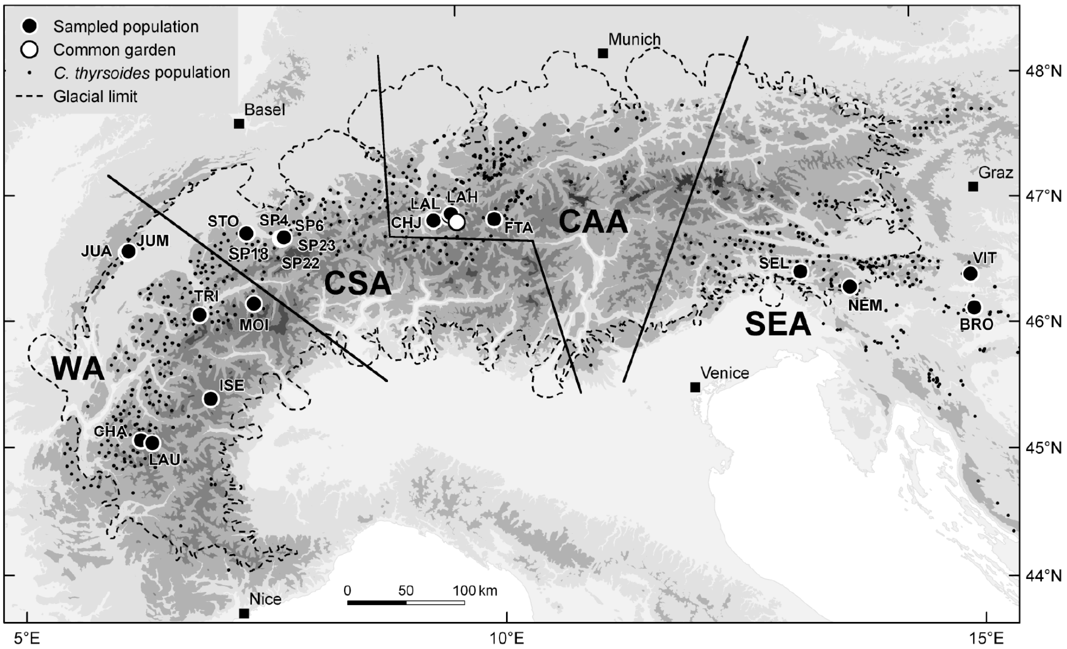


Fig. 1. Map of the European Alps showing the 21 sampled populations in four phylogeographic regions, the common garden location, the distribution of *Campanula thyrsoides* and the glacial limit from the last glacial maximum. Lines delineating the regions are schematic (see Kuss *et al.* 2011). *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

These phylogeographic regions were chosen based on the genetic structure revealed by Bayesian clustering analysis using nuclear microsatellite data (Kuss *et al.* 2011). In this study, SEA populations contain only subsp. *carniolica*, whereas populations from the other three regions contain only subsp. *thyrsoides*. WA includes two populations from the Jura Mts. From September 2007, randomly chosen seeds were germinated on moist filter paper in Petri dishes in a greenhouse located in Basel, Switzerland (276 m a.s.l.). As plants bear around 50–60 flowers (Kuss *et al.* 2007; Scheepens *et al.* 2011) which are pollinated by multiple pollinators (Scheepens *et al.*; unpublished results) and together produce thousands of seeds (Kuss *et al.* 2007), we treated the randomly chosen seeds as half-sibs. Eight seedlings per seed family were planted into pots of 4 cm diameter filled with low-nutrient soil (Anzuchterde, Ökohum, Herrenhof, Switzerland). Plants were repotted after 10–18 weeks into pots of 10 × 10 × 10 cm^3 with potting soil (Topferde, Ökohum, Herrenhof, Switzerland). Insecticide (Traunem, BioControl, Andermatt; Basudin Extra, Novartis Agro, Dielsdorf, Switzerland) was sprayed several times to control Aphidoidea and Sciaridae outbreaks, and fertiliser (Wuxal, Maag, Düsseldorf) was added once. In spring, plants were transferred outside the greenhouse to acclimatise before final transplantation, and anti-snail grains (Ferramol, BioControl, Andermatt) were applied to limit herbivory by snails.

On 19 May 2008, plants were transplanted to a common garden located at 1,530 m a.s.l. in Davos, Graubünden, Switzerland (N 46°47'06.97", E 9°48'57.02"). The site, formerly used as an organically fertilised subalpine meadow-pasture, was ploughed before plants were transplanted into the local soil. Out of 1,008 plants, 953 individuals could be transplanted (Appendix 1) as there were missing individuals in several populations due to mortality in the greenhouse. The plants were distributed among four blocks each with two members of each seed family. Rainfall at the common garden location averages 1,026 mm per year and minimum, mean and maximum temperature are -8.2°C, 2.9°C and 15.1°C respectively (WorldClim data, based on monthly averages; Hijmans *et al.* 2005). The experimental site was fenced and plant beds were regularly weeded.

During transplantation, rosette diameter was measured, which was used as a covariate in all subsequent analyses. Eight weeks after transplantation, on 15 July 2008, a clipping treatment to simulate herbivory was applied to half of the plants (one out of two seed family members in each block). Using scissors we cut off all leaves as close as possible to the rosette center without injuring the apical meristem. At the end of the growing season, on 9 September 2008, leaf length and width of the longest leaf and number of leaves were measured, and leaf length and width of the longest leaf were measured again on 1 June 2009 since oblongate spring leaves are replaced in summer for obovate leaves in this species (Jäger 2000). For each flowering plant, the number of inflorescences, the height and the number of flowers were measured on 27 July 2009, and again on 20 October 2009 for most SEA plants. The above-ground biomass was harvested when plants were ripening and was weighed after drying for 72 hours at 60°C in a drying oven. During each visit, life-history stages were recorded, using the classes *dead*, *rosette*, *bolting* (i.e. initiation of flowering), *flowering* (i.e. at least one flower in anthesis) and *ripening* (i.e. when all flowers were wilted), and from this data post-transplantation survival (i.e. still alive on 15 July 2008) could be deduced.

Generalised linear mixed-effects models

Leaf length to width ratio for 9 September 2008 and for 1 June 2009 were derived from leaf length and width measurements. These derived traits, as well as the number of leaves, number of inflorescences, maximum inflorescence height, number of flowers and above-ground biomass, were analysed using generalised linear mixed-effects models (GLMMs; Crawley 2007). We applied Type I sums of squares, which assesses effects sequentially, allowing the removal of covariate effects before testing remaining factors. The drawback is that the order could affect the results in unbalanced designs, so it is advisable to compare models with different factor sequences (Crawley 2007).

In all models, the rosette diameter at the start of the experiment was included as a covariate to account for effects of initial size on the measured variables. Besides removing potential maternal effects and size differences due to variable germination dates, rosette diameter may remove part of the genetic variation among plants. However, if subsequent factors remain significant, it is all the more indicative for genetic variation in these factors and the results are more reliable. The four blocks, a random factor to remove potential environmental variability within the experimental site, did not show significant differences and were therefore not considered in the models. The clipping treatment was included as a fixed effect in all models, except in the model testing post-transplantation survival, which was assessed before the treatment was applied. Phylogeographic region (fixed), population (random) and seed family (random) were nested in each other, and so were interactions of the clipping treatment with region (fixed), population (random) and seed family (random).

Survival after transplantation was analysed using a binomial error distribution with a logit-link function, and number of inflorescences was analysed using a quasi-Poisson error distribution with a log-link function to overcome overdispersion (Crawley 2007). Number of leaves and number of flowers fitted a normal distribution better than a Poisson distribution, so these and the remaining response variables were analysed with a normal error distribution. For all traits analysed with normal error expectations, the normality of full model residuals and homogeneity of variances were checked visually by constructing diagnostic plots. To improve normality of their model residuals, we used power transformations (Crawley 2007): number of leaves was transformed using the power of 0.656, number of flowers using 0.620 and above-ground biomass using 0.331. The model residuals of maximum inflorescence height violated normality considerably, and transformations only worsened normality, but since non-normality was due to left-skewed data and not due to outliers, untransformed data were used.

To test the significance of model factors, we calculated chi^2-values and *P*-values from likelihood ratio tests of model comparisons using maximum likelihood, starting with the deletion of the interactions and removing factors subsequently until all factors had been tested. Variance component analyses were performed on the full models treating all factors as random and using restricted maximum likelihood (REML; Crawley 2007). Tukey's HSD tests were used to locate differences between region pairs.

Any observed regional differentiation could be an effect of alpine-wide distance-related effects of drift. To test whether regional differentiation remained when this confounding factor was removed, we included the mean Euclidean distance of each focal population to all other populations as covariate in a second set of models, positioned in the model directly after rosette diameter.

Response to clipping

The response to clipping in number of flowers and above-ground biomass was used as a measure of the ability to overcome negative effects of grazing and mowing. The means of clipped and unclipped plants from a single seed family were logarithmised and the former subtracted from the latter to calculate the proportional reduction, which indicates the severity of clipping for that seed family (Pluess & Stöcklin 2004). This value was subtracted from unity to obtain a measure of the relative ability to withstand clipping, where lower values indicate a stronger susceptibility. Regional means were based on population means, which were calculated from seed family means. Tukey's HSD tests were performed to test for significant differences in the ability to withstand grazing between phylogeographic regions.

Adaptation

Pearson's correlations were performed between population-averaged trait values of unclipped plants, calculated from seed family means, and altitude of population origin (Appendix 1), which was used as a proxy for environmental variables related with altitude. Altitude of population origin correlates strongly ($r = 0.68$, $P < 0.001$) with the first principal component of climatic data, which in turn explains 99.97% of the four climatic variables precipitation and minimum, mean and maximum temperature (based on monthly averages, WorldClim data, Hijmans *et al.* 2005; data not shown). To investigate the effect of the two subspecies on the correlation, we tested a subset containing WA, CSA and CAA populations (subsp. *thyrsoides*) as well as a subset with SEA populations (subsp. *carniolica*; Scheepens *et al.* 2010), although the latter contained too few populations ($n = 4$) for significance detection. Significance of groups of correlations was estimated using sequential Holm-Bonferroni corrections.

All analyses were performed using the R statistical package (R Development Core Team 2009; version 2.10.1) with *lmer()* from package LME4 for analysing GLMMs (Bates & Maechler 2009).

Results

Phenotypic differentiation

Significant regional differentiation was present in all traits (Table 1; Fig. 2) and the variance explained by phylogeographic region ranged from 0% (leaf length to width ratio 2009) to 24% (maximum inflorescence height).

Table 1. Results of generalised linear mixed-effects model (GLMM) analysis of the effects of initial rosette diameter (covariate), clipping treatment (fixed), phylogeographic region (fixed), population (random) nested in region, seed family (random) nested in population and interactions of clipping with region (fixed), population (random) and seed family (random) on eight phenotypic traits of *Campanula thyrsoides* in the common garden

		Survival			No. of leaves			Leaf length to width ratio 2008			Leaf length to width ratio 2009		
	df	Chi^2		%*VC*	Chi^2		%*VC*	Chi^2		%*VC*	Chi^2		%*VC*
Initial rosette diameter	1	479.0	***	64.2	156.7	***	16.3	94.3	***	4.0	52.1	***	3.3
Clipping treatment	1	-		-	144.7	***	23.5	1.1		0.2	36.6	***	6.4
Region	3	20.1	***	17.2	69.0	***	2.6	49.7	***	9.0	9.6	*	0.0
Population (Region)	1	2.3		4.1	71.0	***	7.3	108.8	***	17.7	55.2	***	7.5
Seed family (Population)	1	1.0		0.6	4.2	*	2.2	19.5	***	8.4	9.6	**	7.9
Clipping×Region	3	-		-	4.4		0.0	2.4		0.0	2.2		0.0
Clipping×Pop (Region)	1	-		-	0.1		0.4	0.1		0.8	4.3	*	5.5
Clipping×Seed family (Pop)	1	-		-	0.5		2.8	0.0		0.0	0.0		0.0
Residuals	755-942			13.9			44.9			60.0			69.4

		No. of inflorescences			Max. height of inflorescence			No. of flowers			Above-ground biomass		
	df	Chi^2		%*VC*	Chi^2		%*VC*	Chi^2		%*VC*	Chi^2		%*VC*
Initial rosette diameter	1	15.4	***	0.1	52.5	***	0.0	34.9	***	0.3	85.1	***	10.4
Clipping treatment	1	54.4	***	1.6	20.9	***	2.7	93.9	***	21.5	72.8	***	14.8
Region	3	34.9	***	1.0	128.9	***	23.9	24.8	***	0.8	90.0	***	15.9
Population (Region)	1	70.7	***	3.5	81.6	***	13.0	35.0	***	9.9	14.4	***	3.8
Seed family (Population)	1	15.6	***	2.3	19.2	***	9.7	0.0		1.6	4.8	*	4.7
Clipping×Region	3	1.8		0.0	7.8	*	0.0	7.6	(*)	1.0	18.7	***	3.0
Clipping×Pop (Region)	1	0.0		0.0	6.7	**	5.3	0.0		1.9	1.1		1.8
Clipping×Seed family (Pop)	1	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0
Residuals	609-681			91.5			45.4			62.9			45.5

Survival, post-transplantation survival; df, degrees of freedom. Residual df varies per trait due to mortality and due to flowering traits being recorded in flowering plants only. Chi^2 values and their significances were obtained from model comparisons. %*VC*, variance components were obtained from analyses with all factors treated as random effects. (*)$P = 0.054$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Post-transplantation survival was higher in CSA populations compared to WA populations, number of leaves was higher in CAA than in SEA plants, maximum inflorescence height was higher in SEA than in WA plants, and SEA populations showed higher above-ground biomass compared to populations from the other regions (Table 2; Fig. 2).

Table 2. Mean values of morphological traits of *Campanula thyrsoides* per phylogeographic region and treatment (control versus clipped plants) in the common garden

	Region				Treatment	
	WA	CSA	CAA	SEA	Control	Clipped
Post-transplantation survival (%)	76.3^{a} (10.2)	94.5^{b} (3.1)	99.0^{ab} (0.6)	78.7^{ab} (7.4)	-	-
Leaf length to width ratio 2008	2.71 (0.11)	2.62 (0.09)	3.27 (0.24)	3.18 (0.33)	2.92 (0.15)	2.99 (0.16)
Leaf length to width ratio 2009	7.25 (0.42)	6.86 (0.36)	7.26 (0.45)	7.18 (0.57)	7.70^{a} (0.10)	6.77^{b} (0.24)
No. of leaves	106.3^{ab} (9.7)	100.0^{ab} (6.7)	128.3^{a} (9.9)	82.4^{b} (9.1)	126.0^{a} (11.7)	84.1^{b} (7.4)
No. of inflorescences	4.06 (0.55)	4.86 (0.23)	4.65 (0.31)	6.22 (0.87)	5.6^{a} (0.4)	4.3^{b} (0.5)
Max. height of inflorescence (cm)	37.21^{a} (1.26)	38.86^{ab} (2.21)	37.58^{ab} (2.50)	52.33^{b} (4.62)	43.1^{a} (3.8)	39.8^{b} (3.2)
No. of flowers	254.7 (31.0)	285.5 (23.3)	335.3 (33.7)	241.1 (66.4)	347.8^{a} (38.3)	217.9^{b} (10.3)
Above-ground biomass (g)	18.89^{a} (2.67)	20.96^{a} (2.62)	23.15^{a} (4.52)	53.40^{b} (4.44)	38.1^{a} (11.1)	21.9^{b} (5.2)

Means (SE) of regions are based on population means, which in turn are based on seed family means. Different superscript letters indicate significant differences ($\alpha = 5\%$) among regions using Tukey's HSD tests and between treatments based on significance of the treatment factor in the model (Table 1). *WA*, Western Alps; *CSA*, Central Swiss Alps; *CAA*, Central Austrian Alps; *SEA*, Southeastern Alps.

Although region was significant in all models, post-hoc tests could not locate significant differences between regions for leaf length to width ratios, number of inflorescences and number of flowers (Table 2), which can be attributed to low explained variance, especially in relation to the variance explained by populations, as well as to lack of statistical power since only 4–7 populations per region were incorporated in the study.

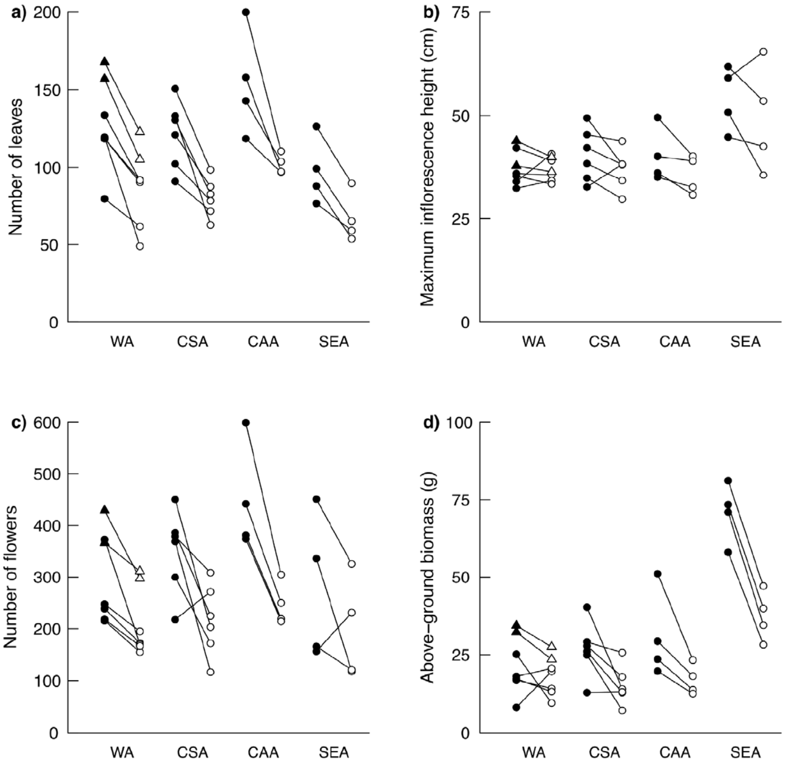


Fig. 2. Reaction norms of populations to the clipping treatment on *Campanula thyrsoides* plants in the common garden ordered by phylogeographic lineage for (a) number of leaves, (b) maximum inflorescence height, (c) number of flowers, and (d) above-ground biomass. Filled circles indicate control plant means, open circles indicate clipped plant means. Triangles indicate the two populations from the Jura Mts. *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

The clipping treatment significantly reduced all trait values except for leaf length to width ratio in 2008 (Tables 2 and 3). A clipping×region interaction was found for maximum inflorescence height, number of flowers (marginally significant) and above-ground biomass, indicating differences among the phylogeogaphic lineages in the response to clipping (Table 1). Several populations responded positively to clipping in maximum inflorescence height, number of flowers and above-ground biomass (Fig. 2). When measured as the proportional seed family reduction due to clipping, significant differences in the response of maximum inflorescence height (data not shown) and number of flowers (Fig. 3a) could not be located among regions, but biomass of plants from CSA and SEA was significantly reduced by clipping compared to plants from WA (Fig. 3b).

Table 3. Correlations between measured plant traits in the common garden and altitude of origin in *Campanula thyrsoides* involving (i) all populations, (ii) WA, CSA and CAA populations ($n = 17$), and (iii) SEA populations ($n = 4$)

	All regions		WA, CSA, CAA		SEA	
	r	P-value	r	P-value	r	P-value
Post-transplantation survival	0.13	0.56	-0.26	0.32	0.79	0.21
Leaf length to width ratio 2008	-0.38	0.088	-0.24	0.36	-0.37	0.63
Leaf length to width ratio 2009	-0.27	0.23	-0.53	0.027	-0.53	0.47
No. of leaves	0.26	0.26	-0.46	0.061	-0.13	0.87
No. of inflorescences	-0.70	0.00039 *	-0.64	0.0052	-0.73	0.27
Max. height of inflorescence	-0.82	$5.1 \bullet 10^{-6}$ ***	-0.46	0.064	-0.94	0.061
No. of flowers	0.04	0.85	-0.50	0.042	-0.55	0.45
Above-ground biomass	-0.93	$1.0 \bullet 10^{-9}$ ***	-0.61	0.010	-0.97	0.031

r, Pearson's correlation coefficient. Sequential Holm-Bonferroni corrected P-values referring to: $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

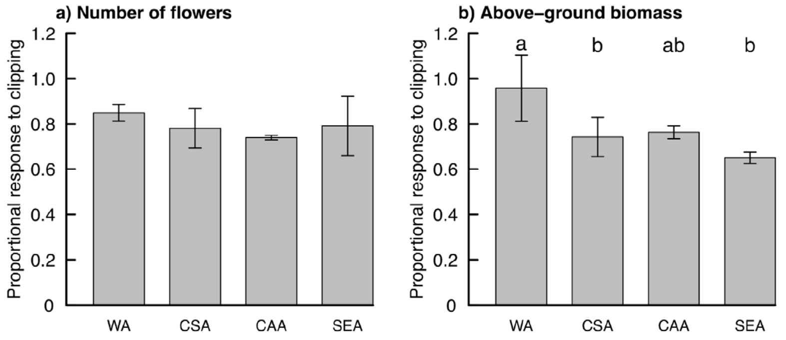


Fig. 3. Mean proportional response to clipping per phylogeographic lineage of *Campanula thyrosides* plants in the common garden for (a) number of flowers, and (b) above-ground biomass. Means ± 1 SE are shown. Letters denote significant differences ($\alpha = 5\%$) obtained with a Tukey's HSD test. *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

Populations within regions were significantly different for most traits (except for survival) and explained more variation than phylogeographic region in number of leaves, leaf length to width ratio in 2008 and 2009 as well as in number of inflorescences and number of flowers (Table 1). Noteworthy is that the two populations from the Jura generally had higher values for number of leaves and above-ground biomass compared to other WA populations (Fig. 2). The Jura populations also reached numbers of inflorescences similar to those of SEA populations, whereas the remaining WA populations had the lowest numbers (data not shown). Seed families within populations were significant for all traits except survival and number of flowers, and explained considerable amounts of variation (Table 1). There were significant clipping×population interactions for leaf length to width ratio in 2009 and maximum inflorescence height (Table 1), which indicate genetic differences in strength and direction of the response to grazing among populations. Clipping×seed family interactions were never significant, indicating that all seed families within a population responded equally to the clipping treatment.

Rosette diameter at the start of the experiment affected the outcome of all variables significantly, but the amount of variance explained by this factor was highly variable (Table 1). It affected survival after transplantation strongly (64%) and positively, and had a substantial effect on number of leaves (16%) and biomass (10%). Variation in reproductive traits explained by initial rosette diameter was negligible (0–0.3%). Due to considerable orthogonality in the design, changing the position of factors while respecting the nesting structure and as long as interactions did not precede main factors contained in these interactions, had only negligable influence on the results (results not shown).

By including the mean Euclidean distance of each focal population to all other populations as covariate in the models, we excluded distance effects on drift among populations across the European Alps. By doing so, highly significant regional differentiation was retained for all traits (Appendix 2). Including this distance factor did not influence other factors qualitatively.

Out of the total 953 plants, 132 individuals died between transplantation and the second measurement. This post-transplantation survival was significantly dependent on phylogeographic region, explaining 17% of variation, as WA and SEA had less surviving plants compared to the two central regions (Tables 1 and 2). Only fifteen plants died between the second and third measurement in the first season, only 35 plants died over winter and no plants died between the first and second measurement of the second season.

At the first measurement of 2009, the majority of surviving plants of WA, CSA and CAA had already started bolting, whereas SEA plants showed no sign of initiation of flowering (Fig. 4). At the second measurement of 2009, the majority of surviving plants from WA, Plants from CSA and CAA were ripening, whereas only 7% of SEA plants reached the ripening stage and 83% were flowering (Fig. 4). Most SEA plants had finished flowering only on 20 October 2009, when snow and frost hampered further growth.

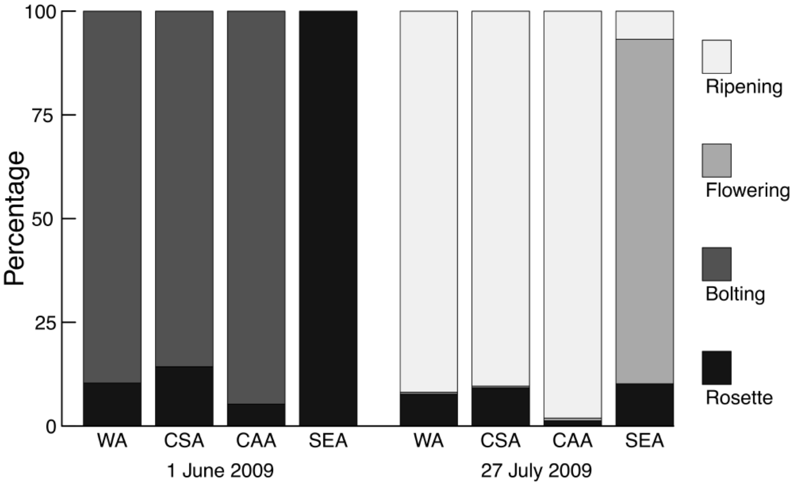


Fig. 4. Percentage of plants in four distinct phenological stages (rosette, bolting, flowering, ripening) of surviving *Campanula thyrsoides* plants in the common garden from four phylogeographic regions at two census dates. *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

Local adaptation

Correlations between trait values measured in the common garden and altitude of origin were significant for the number of inflorescences, maximum inflorescence height and above-ground biomass (Table 3). For WA, CSA and CAA populations, only the number of inflorescences retained significance. The number of SEA populations was too low to test significance of the correlations but the number of inflorescences and above-ground biomass had very high Pearson's correlation coefficients.

Discussion

Phenotypic differentiation

The detected regional differentiation in vegetative and reproductive traits (Tables 1 and 2, Fig. 2) as well as in the response to clipping (Fig. 3) and in flowering phenology (Fig. 4) in *Campanula thyrsoides* is in line with the observed spatial genetic structure based on nuclear microsatellite data which previously confirmed four longitudinally oriented phylogeographic regions (Kuss *et al.* 2011). After including the mean Euclidean distance of each focal population to all other populations as a covariate in the models, region remained significant (Appendix 2). These results therefore support our hypothesis that glacial history caused regional differentiation in several phenotypic traits across the Alps in this species, and that a potential confounding effect of regional differentiation due to relatively recent distance-related effects of drift is unlikely. This regional differentiation could be caused by drift or by regional adaptation during survival in glacial refugia or during recolonisation after the retreat of glaciers. Due to the generally strong isolation of *C. thyrsoides* populations and the limited insect-mediated pollen dispersal, gene flow between populations is probably weak, which may have prevented homogenisation of phenotypic differentiation across the phylogeographic regions.

Regional adaptation

Differentiation in phenotypic traits can evolve due to genetic drift or adaptation, or a combination of both. Adaptation to regional conditions could have played a role in the observed differentiation of investigated traits. A clear example is the delayed flowering of SEA plants (ssp. *carniolica*; Fig. 4), which is adaptive to the long submediterranean summers, compared to the earlier flowering optimal for plants from the other regions (ssp. *thyrsoides*) growing at higher altitude (Scheepens *et al.* 2011). For plants experiencing a long growing season, delayed flowering allows for prolonged build-up of reserves in spring and therefore results in higher seed output, whereas a short growing season selects for early onset of flowering and rapid fulfillment of the life cycle (Rathcke & Lacey 1985; Weber & Schmid 1998;

Olsson & Ågren 2002; Sandring *et al.* 2007). Delayed flowering was also observed in native populations of SEA, thereby confirming that the observed delay is to a large extent genetically determined and not merely a response to the common garden environment (Scheepens *et al.* 2011).

An ultimate cause for the strong morphological and phenological differentiation of SEA populations versus WA, CSA and CAA populations could be that the Southeastern lineage of *C. thyrsoides* has survived the glaciation in situ under environmental conditions contrasting with those lineages in the other regions where populations probably survived ex situ in refugia along the northern fringe of the Alps, causing differential adaptation to their respective climates (Jäger 2000; Kuss *et al.* 2007, 2011; Scheepens *et al.* 2010, 2011). This may have caused the current distribution, with colline-montane populations from the Southeastern Alps experiencing a prolonged season compared to subalpine-alpine populations from the other regions. The distributional and reproductive isolation in the two subspecies could finally lead towards glacial history-driven allopatric speciation (Scheepens *et al.* 2011).

Response to clipping

Although many studies investigated the effect of herbivory on growth per se (e.g. Escarré *et al.* 1996), and quite some studies established genetic phenotypic differences between populations from sites with different long-term grazing regimes (e.g. Warwick & Briggs 1979; Zopfi 1993; Suzuki 2008), few have investigated whether the response to clipping differed between populations with (potentially) different grazing regimes. To our knowledge, studies so far only detected absence of differentiation in the response to clipping (Rotundo & Aguiar 2008; Suzuki 2008).

In this study, plants from different regions responded differently to the clipping treatment (Table 1). WA plants were less affected by clipping compared to plants from other regions, where clipping generally had negative effects on traits (Figs 2 and 3). Some populations even benefitted from clipping (i.e. overcompensation; McNaughton 1983), leading to clipping×population interaction for leaf length to width ratio in 2009 and maximum inflorescence height (Table 1; Fig. 2). *C. thyrsoides* often occurs on the intersection of steep slope grassland to screes, where competition and grazing are limited and where slope movement creates microsites for germination (Kuss *et al.* 2007). However, the Western Alps including the Jura Mts. is the region where *C. thyrsoides* occurs most pronouncedly in pastures and meadows (Jürg Stöcklin, pers. obs.), thus potentially being subject to regular grazing or mowing. Although data on grazing pressure in the investigated populations, are lacking, which would allow more reliable tests, the results of this study indicate regional differentiation in susceptibility to clipping, which may explain the respective habitat preference in this region. It remains unclear whether this supposedly adaptive differentiation is the result of glacial history or recent selection.

Population level differentiation

Populations within regions were differentiated for all measured traits except for survival (Table 2). This population differentiation could be due to neutral genetic drift as populations can have small size and can thus be subject to considerable drift (Stöcklin *et al.* 2009). However, adaptation to the population-specific environment could also have played a role (Joshi *et al.* 2001; Ramírez-Valiente *et al.* 2009) as the Alpine landscape is spatially and temporally heterogeneous (Körner 2003). Since variance explained by population was higher than by phylogeographic region in five out of eight traits (Table 1), it could be argued that population-specific local conditions have had more influence on postglacial adaptations than effects of glacial history had on phylogeographic differentiation, but the relationship could of course also reflect the effects of time on eroding historical signatures.

The two populations from the Jura Mts., although belonging phylogenetically to the Western Alps and thus likely originating from the same refugium (Ægisdóttir *et al.* 2009; Kuss *et al.* 2011), stood out with higher number of leaves, number of flowers, biomass (Fig. 2) and number of inflorescences (data not shown) compared to other WA populations. This suggests that these populations from the Jura Mts. could be considered to belong to a phenotypically distinct group which has adapted to the specific environmental conditions in the Jura Mts. Climatic differences, e.g. the longer snow-free season at this comparably lower altitude, could have led to the increased size of traits. The different vegetation communities in which *C. thyrsoides* occurs in the Jura Mts. compared to the Alps (Kuss *et al.* 2007), may also pose different selection pressures.

Seed families were differentiated but responded similarly to the clipping treatment (Table 1), which suggests that whole populations may have adapted to grazing or mowing and that the above results are not due to few grazing-resistant seed families.

Initial rosette diameter and maternal effects

The plants were ca six months old when transplantated to the common garden and most traits were measured > 2.5 months after transplantation. Small initial differences in the rosette diameter of *C. thyrsoides* influenced early survival and vegetative traits during the first season, as well as final biomass, whereas reproductive output, measured in the second season, remained unaffected (Table 1). These results may be partly due to maternal effects, and they suggest that the influence of maternal effects diminish over time, as has been found in other studies (e.g. Ouborg *et al.* 1991; Schmid & Dolt 1994). Therefore we assume that maternal effects are negligable, especially for reproductive traits which are related most strongly to fitness.

Local adaptation

The negative correlations between altitude of origin and number of inflorescences, maximum inflorescence height and above-ground biomass measured in the common garden (Table 3) suggest local adaptation to climatic variables related to altitude across the European Alps. It is known that forbs invest less in stem mass and more in fine roots with increasing altitude (Körner & Renhardt 1987), but the functional explanation of this pattern is still unclear (Körner 2003). The inflorescence of *C. thyrsoides* determines both height and the larger part of the above-ground biomass. Our results therefore suggest that the widely-observed pattern in forbs may also apply to the inflorescence of monocarpic species. The three traits correlating with altitude are clearly also correlated with each other, reflecting a decreased energy budget with increasing altitude.

Populations from the SEA region (subsp. *carniolica*) occur at much lower altitude compared to populations from the other regions (subsp. *thyrsoides*), which could bias the correlation analysis as these two groups are climatically so different that gradients in phenotypic traits could be disrupted (Scheepens *et al.* 2010); this may be the case in the number of leaves and number of flowers, which show negative correlation coefficients for the analyses of the separate subspecies (Table 3). When correlating WA, CSA and CAA populations (subsp. *thyrsoides*) with altitude of origin, only the number of inflorescences retained its significance (Table 3). No sigificant correlations were present for the SEA populations, which could be due to the low number of populations ($n = 4$), but Pearson's correlation coefficients for maximum inflorescence height and above-ground biomass remained negative and strong in both subspecies.

Monty and Mahy (2009) found similar negative relationships for final height and above-ground biomass in a common garden experiment with *Senecio inaequidens* originating from two contrasting altitudinal transects from northern Belgium and the French Pyrenees. Plant height was also decreasing with altitude of origin in a study on *Festuca eskia* by Gonzalo-Turpin and Hazard (2009), and plant size and vegetative and reproductive investment decreased with altitude of origin in the alpine fodder grass *Poa alpina* (Hautier *et al.* 2009). Decreasing size with increasing altitude has been explained as an adaptation to harsher conditions and shorter growing seasons (Galen *et al.* 1991; Körner 2003).

Conclusions

We showed that regional phenotypic differentiation in an Alpine plant is in line with neutral molecular differentiation, suggesting that glacial history is responsible for this phenotypic differentiation and that the four phylogeographic lineages diverged independently by drift and selection in the past. Glacial history can therefore have a strong and long-lasting influence on the phenotypic evolution of Alpine plants and could be a driver of allopatric speciation. Although postglacial local adaptation to the heterogeneous landscape of the Alps was apparent from several trait correlations with altitude of origin, this proved insufficient to erase the signature of glacial history.

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Appendix

Appendix 1. Location, geographic coordinates (WGS 84) and altitude (m a.s.l.) of 21 sampled *Campanula thyrsoides* populations across the Alps and Jura Mts.

Location	Code	Region	Northing	Easting	Altitude	*n*
Jura, Les Amburnez	JUA	WA	46°32'27.52"	6°13'58.57"	1340	48
Jura, Col du Marchairuz	JUM	WA	46°33'06.27"	6°15'13.46"	1440	47
Le Chazelet	CHA	WA	45°03'23.51"	6°16'55.49"	1757	46
Col du Lautaret	LAU	WA	45°02'03.17"	6°23'59.63"	2025	47
Trient, Les Tseppes	TRI	WA	46°02'53.93"	6°58'47.05"	2020	48
Col d'Iseran	ISE	WA	45°23'10.42"	7°02'50.81"	2212	48
Lac du Moiry	MOI	WA	46°08'12.78"	7°34'02.87"	2266	48
Stockhorn	STO	CSA	46°41'37.05"	7°32'17.05"	2148	38
Schynige Platte 4	SP4	CSA	46°39'17.31"	7°54'16.67"	1911	48
Schynige Platte 6	SP6	CSA	46°39'15.23"	7°54'19.79"	1916	48
Schynige Platte 18	SP18	CSA	46°39'33.73"	7°55'14.41"	1930	48
Schynige Platte 22	SP22	CSA	46°39'46.86"	7°55'57.57"	2022	44
Schynige Platte 23	SP23	CSA	46°39'46.12"	7°56'14.21"	1958	49
Churwalden, Joch	CHJ	CAA	46°47'51.41"	9°33'53.65"	1890	48
Langwies, Holzbüel	LAH	CAA	46°49'41.97"	9°44'00.53"	1700	42
Langwies, Listboden	LAL	CAA	46°51'07.02"	9°45'32.22"	2000	48
Ftan, Prui	FTA	CAA	46°48'32.68"	10°13'20.37"	2101	49
Sella Nevea	SEL	SEA	46°23'35.00"	13°27'46.00"	932	28
Nemski Rovt	NEM	SEA	46°16'23.50"	13°58'30.00"	663	49
Brodnice	BRO	SEA	46°06'24.30"	15°16'53.10"	283	42
Vitanje	VIT	SEA	46°22'27.80"	15°17'16.90"	422	40

Code, abbreviation as used in the main text; Region, phylogeographic region: *WA* Western Alps (including Jura Mts.); *CSA* Central Swiss Alps; *CAA* Central Austrian Alps; *SEA* Southeastern Alps; *n*, sample size of individuals used in the common garden. Region according to Kuss *et al.* (2011).

Appendix 2. Results of generalised linear mixed-effects model (GLMM) analysis of the effects of initial rosette diameter (covariate), mean Euclidean distance of each focal population to all other populations (covariate), clipping treatment (fixed), phylogeographic region (fixed), population (random) nested in region, seed family (random) nested in population and interactions of clipping with region (fixed), population (random) and seed family (random) on eight phenotypic traits of *Campanula thyrsoides* in the common garden

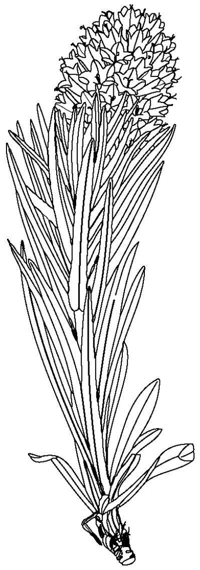
		Survival			No. of leaves			Leaf length to width ratio 2008			Leaf length to width ratio 2009		
	df	Chi^2		*%VC*	Chi^2		*%VC*	Chi^2		*%VC*	Chi^2		*%VC*
Initial rosette diameter	1	479.0	***	62.8	156.7	***	16.3	94.3	***	4.0	52.1	***	3.3
Distance	1	0.0		2.6	43.4	***	3.6	14.7	***	8.9	0.3		3.7
Clipping treatment	1	-		-	155.9	***	23.5	1.2		0.2	36.7	***	6.4
Region	3	24.0	***	18.3	17.1	***	2.6	35.0	***	9.0	19.9	***	0.0
Population (Region)	1	0.4		2.6	68.8	***	3.6	108.9	***	8.9	46.8	***	3.7
Seed family (Population)	1	0.6		0.5	4.1	*	2.2	19.4	***	8.4	9.2	**	7.9
Clipping×Region	3	-		-	4.4		0.0	2.4		0.0	2.2		0.0
Clipping×Pop (Region)	1	-		-	0.1		0.4	0.1		0.8	4.4	*	5.5
Clipping×Seed family (Pop)	1	-		-	0.5		2.8	0.0		0.0	0.0		0.0
Residuals	755-942			13.1			44.9			60.0			69.4
		No. of inflorescences			Max. height of inflorescence			No. of flowers			Above-ground biomass		
	df	Chi^2		*%VC*	Chi^2		*%VC*	Chi^2		*%VC*	Chi^2		*%VC*
Initial rosette diameter	1	15.4	***	0.1	52.5	***	0.0	34.9	***	0.3	85.1	***	10.4
Distance	1	41.2	***	1.9	117.7	***	6.6	0.8		4.0	51.4	***	1.9
Clipping treatment	1	49.8	***	1.6	19.5	***	2.7	95.5	***	21.5	77.8	***	14.8
Region	3	37.3	***	1.0	67.5	***	23.9	43.9	***	0.8	39.8	***	15.9
Population (Region)	1	38.7	***	1.6	39.0	***	6.5	18.6	***	5.9	10.6	**	1.9
Seed family (Population)	1	16.0	***	2.3	18.1	***	9.7	0.0		1.6	4.6	*	4.7
Clipping×Region	3	1.8		0.0	7.6	(*)	0.0	7.7	(*)	1.0	18.7	***	3.0
Clipping×Pop (Region)	1	0.0		0.0	7.1	**	5.3	0.0		1.9	1.1		1.8
Clipping×Seed family (Pop)	1	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0
Residuals	609-681			91.5			45.4			62.9			45.5

Survival, post-transplantation survival; df, degrees of freedom. Residual df varies per trait due to mortality and due to flowering traits being recorded in flowering plants only. *%VC*, variance components were obtained from analyses with all factors treated as random effects. Chi^2 values and their significances were obtained from model comparisons. (*)$P = 0.054$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Chapter 4

High genetic differentiation and founder effects in populations of a rare Alpine plant on a small mountain plateau

Eva S. Frei, J.F. Scheepens & Jürg Stöcklin



Summary

- *Premise of the study*: How gene flow determines population persistence in a small region is important for conservation of rare plant species when habitats are shrinking due to natural or man-made fragmentation of the landscape. In the European Alps, the evolutionary processes shaping the genetic structure of Alpine plants, particularly at a small spatial scale, are not well understood. Here, we investigate the genetic differentiation of a rare Alpine plant on a small and highly fragmented mountain plateau in the Swiss Alps.
- *Methods*: Using microsatellites we investigated genetic differentiation among and within 24 populations of the bell flower *Campanula thyrsoides*. We combined traditional *F*-Statistics with Bayesian clustering analyses and tessellation methods to infer spatial genetic structure. We also compared our results with previous findings observed in this species at the much larger scales of the Swiss Alps and the entire Alpine range.
- *Key results*: The genetic diversity ($H_e = 0.71$) and differentiation ($G'_{ST} = 0.32$) was remarkably high. We detected a pronounced irregular spatial structure of pairwise genetic differentiation. Genetic bottlenecks in several populations indicated founder effects.
- *Conclusions*: Drift and occasional long-distance seed dispersal are more important than isolation by distance for shaping the spatial genetic structure of *C. thyrsoides* at small local scale. Results suggest that occasional gene flow and overlapping generations are sufficient to overcome negative effects of bottlenecks in this monocarpic species. We conclude that the rare bell flower is not endangered on this mountain plateau despite the small size and geographical isolation of its populations.

Key-words: *Campanula thyrsoides*, dispersal ability, European Alps, genetic bottlenecks, gene flow, landscape approach, microsatellites, spatial genetic structure

Introduction

The study of genetic structure in a landscape context has the potential to largely improve our understanding of how topography influences gene flow and population differentiation in plant species (Sork *et al.* 1999; Manel *et al.* 2003). From the perspective of preserving biodiversity, findings of how gene flow determines population persistence in a small region is important for conservation of rare plant species, especially since habitats of numerous species get smaller due to natural or man-made fragmentation of the landscape (Young *et al.* 1996). For instance, in the European Alps the loss of suitable habitats for numerous plant species is accelerated because of landuse changes (Rudmann-Maurer *et al.* 2008). In addition, the loss of habitats at low elevations and range shifts to higher elevations were already observed in several Alpine plant species (e.g. Frei *et al.* 2010).

Gene flow through pollen or seeds, neutral genetic drift and natural selection are among the most important evolutionary forces shaping genetic structure of populations at different spatial scales (Loveless & Hamrick 1984). Moreover, gene flow is mostly dependent on the landscape structure, breeding system, pollination vectors and seed adaptations for dispersal (Kalisz *et al.* 2001; Gaudeul *et al.* 2007; Yan *et al.* 2009). At the larger scale in the Alps, numerous studies detected a high genetic differentiation and a distinct spatial genetic structure in Alpine plant species (Schönswetter *et al.* 2005; Kuss *et al.* 2008a; Alvarez *et al.* 2009). But so far, genetic differentiation at a small local scale in the Alps has rarely been investigated (e.g. Gaudeul & Till-Bottraud 2008).

The naturally fragmented landscape of the Alps offers an excellent opportunity to study the effect of small-scale variation in topography, exposition, and isolation over short distances on genetic differentiation and spatial structure of plant populations (Till-Bottraud & Gaudeul 2002). In structured alpine landscapes, such as a glacier foreland or a mountain plateau, high genetic differentiation among isolated populations might be expected as a result of random drift, restricted gene flow or selection due to large environmental variation over short distances (Hirao & Kudo 2004; Pluess & Stöcklin 2004). Genetic bottlenecks are likely, because only a few individuals may have colonised unoccupied habitats and, in addition, genetic exchange among established habitats is restricted (Wade & MCCauley 1988). Moreover, as a consequence of such small population sizes, inbreeding due to a loss of genetic diversity might occur (Ellstrand & Elam 1993). In recent years, the notion that populations in fragmented landscapes are highly differentiated genetically has been challenged (Jacquemyn *et al.* 2004; He *et al.* 2010). Low genetic differentiation among isolated populations is sometimes explained with more frequent long-distance dispersal as previously assumed and dispersal through seeds is assumed to be more important than dispersal through pollen (Bacles *et al.* 2006; Yang *et al.* 2008).

Since gene flow is more likely among geographically close populations, isolation by distance is expected to create a spatial genetic structure (Hutchison & Templeton 1999), which has been found frequently in the Alps (Pluess & Stöcklin 2004; Gaudeul 2006; Kuss *et al.* 2008a). The few Alpine studies focussing on isolation by distance patterns at different spatial scales were inconsistent, with results showing significant isolation by distance at the small but not at large scale (Stehlik 2002) or by finding the contrary (Gaudeul *et al.* 2000). In addition, the relevance of isolation by distance at a small spatial scale has been questioned (Ennos 2001) and factors like founder effects or snowmelt timing have been shown to be more relevant for genetic structure in studies with a focus on small-scale patterns (Hirao & Kudo 2004; López *et al.* 2010).

Here, we investigated the genetic structure, genetic diversity and inbreeding in 24 spatially isolated populations of the rare Alpine plant *Campanula thyrsoides* L. on a small (10 km^2) mountain plateau in the Swiss Alps (Schynige Platte, Fig. 1A). The landscape is human-altered since the plateau is used as pasture for cattle during summer. The species occurs in numerous and mostly small populations in a mosaic of highly fragmented (semi-) natural habitats such as grasslands and screes separated by forest patches (Fig. 1B).

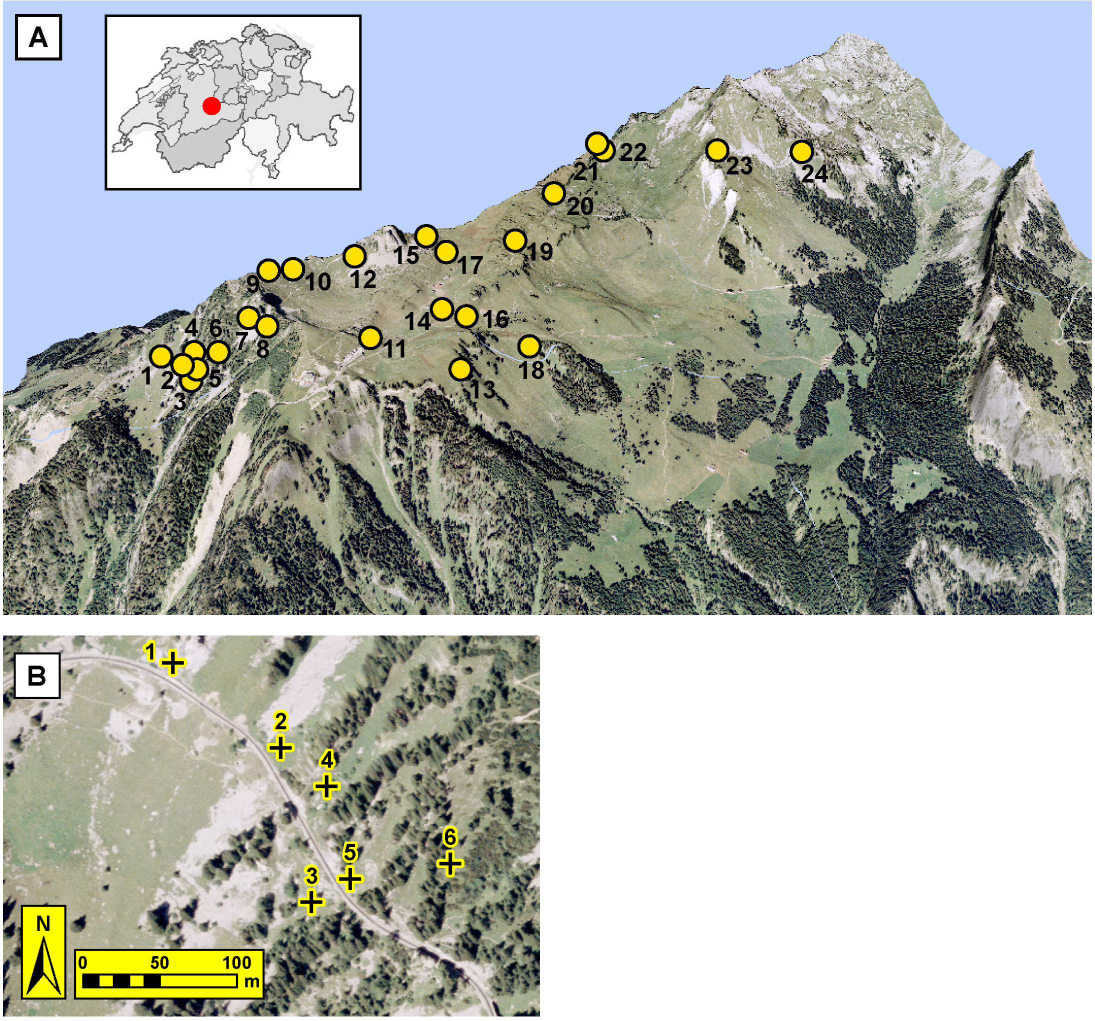


Fig. 1. Study region on the mountain plateau of the Schynige Platte located in the Swiss Alps with (A) all studied 24 populations of *Campanula thyrsoides* and (B) a cut-out of the study region with six populations of close proximity. For geographical distances among populations see Appendix 3. Reproduced by the permission of Swisstopo, Berne, Switzerland (BA100596).

For exclusion of selection as a putative source of differentiation (e.g. Prentice *et al.* 2006), we selected microsatellites as neutral molecular markers. We combined traditional *F*-Statistics with more recent spatial genetic tools such as Bayesian clustering analyses and tessellation methods to infer genetic structure and to test for isolation by distance. Ecological field data from a four-year monitoring period were used to analyse relationships between genetic diversity and population characteristics. The findings of our small-scale study are compared with previous results from *C. thyrsoides* based on microsatellite data at the larger scale of the Central Swiss Alps (18,000 km^2) and the entire Alpine range (190,000 km^2)

(J. Stöcklin, University of Basel, unpublished data). Finally, because *C. thyrsoides* is considered a rare species, we wanted to find out wheter results may have some implications for conservation management. The following questions were addressed: (i) How large is genetic differentiation among 24 populations of *C. thyrsoides* in a small area of 10 km^2 on an isolated mountain plateau? (ii) If differentiation is present, is it distance-dependent (isolation by distance) or otherwise spatially structured? (iii) Is there evidence of founder effects (i.e. genetic bottlenecks) and inbreeding in small populations?

Materials and methods

Study species

Campanula thyrsoides is a bell flower native to the European Alps, the adjacent Dinaric Alps, and Jura Mountains (Kuss *et al.* 2007). Its altitudinal distribution usually ranges from 1,000–2,900m a.s.l. (Aeschimann *et al.* 2004). The species is considered a rare plant, but it is sometimes locally abundant (Kuss *et al.* 2008a). In some countries, like Switzerland, *C. thyrsoides* is red-listed (e.g. Moser *et al.* 2002).

The perennial *C. thyrsoides* is one of the few Alpine monocarpic plants and flowering age ranges from 3–16 years with an average of 10 years (Kuss e*t al.* 2008b). Fifty to 200 yellow and bell-shaped flowers are arranged in a dense spike (Kuss *et al.* 2007). This outcrossing species is self-incompatible but sister-mating is possible (Ægisdóttir *et al.* 2007a). It is mainly pollinated by bumblebees and short pollen dispersal distances are common (Ægisdóttir *et al.* 2009). Seed dispersal occurs primarily by wind, rain, or animals shaking the seeds out of the capsules. Seed dispersal spectra obtained from simulations showed that most seeds (> 99%) are dispersed within a distance of 10 m from the mother plant and that only about 15 seeds per plant are likely to be dispersed over 1 km (Kuss *et al.* 2007).

Field monitoring

The study site is the Schynige Platte, a small mountain plateau (centered at 46° 39’ 12”N; 7° 54’ 42”E) in the northern Swiss Alps consisting of calcareous bedrock (Lüdi 1948). The region covers an area of about 10 km^2 (3 x 3 km) and includes an altitudinal range of 1,800–2,100 m a.s.l. Part of the region is used as summer pasture for cattle since at least 60 years (Lüdi 1948) but probably for centuries. In August 2005, we mapped all, 24, accessible and spatially separated populations of *C. thyrsoides* (Fig. 1). This included one population situated within a Botanical Garden. In June 2006, we estimated the total size of each population either by counting all non-flowering (rosettes) and flowering individuals, or in each of the larger populations, we extrapolated the average number of individuals counted in five sub-plots. In addition to population size, we measured altitude, exposition, slope, occupied area, vegetation cover, and plant density (Table 1). The number of flowering individuals was counted during peak flowering in each summer of the subsequent four years.

Table 1. Location of 24 populations of *Campanula thyrsoides* from the mountain plateau of the Schynige Platte in the Swiss Alps with population characteristics

Population	Lat. °N (Swiss Grid)	Long. °E (Swiss Grid)	Altitude (m a.s.l.)	Exp.	Slope (°)	Area (m^2)	Cover (%)	Density (per m^2)	Size	No. flowering
1	167230	635610	1885	SW	45	60	60	0.50	30	7 (3–12)
2	167175	635680	1885	SW	55	120	95	0.83	100	10 (3–15)
3	**167075**	**635700**	**1900**	**SW**	**45**	**300**	**95**	**1.00**	**300**	**9 (5–10)**
4	167150	635710	1900	SW	50	150	95	1.60	240	32 (23-51)
5	167090	635725	1900	SW	50	200	95	1.00	200	16 (2–20)
6	167100	635790	1950	SW	40	300	90	0.14	42	8 (5–9)
7	167150	635900	2010	W	45	150	95	0.13	20	6 (1–8)
8	167090	635940	2000	W	45	25	85	5.60	140	15 (10–19)
9	167450	635975	2040	S	35	25	70	0.48	12	2 (2–4)
10	167475	636050	2020	S	35	75	90	0.33	25	7 (6–13)
11*	**167000**	**636240**	**1980**	**E**	**40**	**2500**	**95**	**0.16**	**400**	**71 (50–100)**
12	167600	636275	1980	SW	30	3000	90	0.15	460	40 (30–50)
13	166850	636490	1900	SE	30	50	85	3.00	150	9 (5–15)
14	167200	636500	1925	SE	40	700	87	1.00	700	105 (69–167)
15	167750	636540	1960	E	50	300	90	0.10	30	5 (4–10)
16	167175	636575	1910	SE	25	400	92	0.63	250	34 (24–48)
17	167630	636590	1930	SE	50	500	90	0.12	60	16 (4–30)
18	167040	636750	1840	S	65	400	85	0.08	30	16 (4–40)
19	167650	636850	1950	SE	30	1000	95	0.30	300	58 (28–105)
20	168030	637090	1940	SE	15	800	97	0.98	780	57 (9–100)
21	**168430**	**637400**	**2010**	**SE**	**25**	**300**	**90**	**0.57**	**170**	**32 (24–40)**
22	**168425**	**637410**	**1970**	**SE**	**40**	**3000**	**90**	**0.13**	**380**	**75 (35–146)**
23	168125	637800	2030	SE	40	200	85	0.75	150	30 (26–35)
24	168125	638175	1980	S	45	4000	90	0.01	40	6 (0–8)
Mean			1950		40	773	89	0.82	209	28
SD			53		11	1140	6	1.24	212	27

All population characteristics were assessed in 2006 with the exception of number of flowering individuals which shows the mean and the range from the years 2005–2009. Populations are ordered from west to east. Bold values indicate bottlenecked populations (see Table 4). *Site lies within the Botanical Garden. Exp., exposition; Area, area occupied by a population; Cover, coverage of herb layer; Density, plant density; Size, population size (number of flowering and non-flowering individuals); No. flowering, number of flowering individuals.

Molecular analysis

In 2006, we sampled leaf material of 12 individuals within each population, equalling up to 288 individuals. Individuals were sampled randomly within a population and, if possible, separated by at least 3 meters. DNA was extracted from 2 mg of silica-gel dried leaf tissue using a NucleoSpin 96 Plant II extraction kit according to the standard protocol of the manufacturer (Macherey-Nagel GmbH, Düren, Germany). We quantified the DNA concentration with a NanoDrop ND-1000 spectrophotometer (Witec AG, Littau, Switzerland).

We selected microsatellites (SSRs) as neutral molecular markers to reach high enough resolution for detection of the smallest genetic differences among individuals in such a small region (Luikart & England 1999; Selkoe & Toonen 2006; Vandepitte *et al.* 2007). In a pre-analysis, we tested eight microsatellites developed for *C. thyrsoides* (Ægisdóttir *et al.* 2007b) and from there, we selected five that reached highest reproducibility for the final analysis (for details see Appendix 1).

The PCR mixture (10 µL reaction volume) contained 1x PCR buffer including $MgCl_2$ (Qiagen, Hombrechtikon, Schweiz), 150 µM dNTPs (Sigma-Aldrich Chemie GmbH, Buchs, Schweiz), 5 µM each of the forward and reverse primer (Ecogenics, Zürich-Schlieren, Switzerland), ddH_20, 0.5 U polymerase (HotstarTaq polymerase, Qiagen, Hombrechtikon, Schweiz), and 5 ng genomic DNA. PCR amplifications were always run in the same thermal cycler (Techne TC-412, Witec AG, Littau, Switzerland) with the following conditions: 15 min at 95°C for initial denaturation, followed by 38 cycles of optimal annealing temperature for 30 s, 72°C for 30 s and 95°C for 30 s. The PCR finished with a step of 60 s at the optimal annealing temperature and a final extension of 72°C for 30 min. After amplification, the PCR products were separated using a submerged gel apparatus SEA-2000 (Elchrom Scientific AG, Cham, Switzerland) with an electrical field of 120 V. Depending on the primer pair, gels were run between 110–130 min at a temperature of 55°C. Gels were stained for 30 min in ethidium bromide.

We visualised the banding pattern under ultraviolet light using an AlphaDigiDoc photo system (Alpha Innotech Corporation, San Leandro, California, USA). The alleles were scored automatically and the fragment length was estimated using the program IMAGE QUANT TL (GE Healthcare, Buckinghamshire, UK). Scoring was checked manually and all ambiguous PCR results (smear and stutter bands) were repeated to minimise scoring errors. For repeatability of the banding pattern, we performed negative controls in DNA amplification and estimated the error rate by repeating complete PCR analysis for 60 blind samples as proposed by Bonin *et al.* (2004). The resulting error rate was 2.2%.

Genetic differentiation

To estimate genetic differentiation among all populations, we calculated Wright's F_{ST} (Weir & Cockerham 1984) and 95% confidence intervals using the program GENETIX version 4.05 (Belkhir *et al.* 2004). G_{ST} (Nei 1973) and 95% confidence intervals were obtained with 1,000 bootstrap resamplings using the statistical package DEMEtics (Jueterbock *et al.* 2011) in R version 2.12.1 (R Development Core Team 2010). The standardized G'_{ST} was developed to cope with the problem of high values of heterozygosity in highly polymorphic markers like microsatellites (Heller & Siegismund 2009). We calculated G'_{ST} according to equation 4b in Hedrick (2005).

Genetic structure and spatial analyses

To investigate the genetic structure of populations, we used Bayesian structuring analyses. For the assignment of individuals to genetic clusters, we used the program STRUCTURE version 2.3 (Hubisz *et al.* 2009). We selected the "admixture" model with independent allele frequencies (Pritchard *et al.* 2000), but preliminary analyses with the alternative model options yielded highly similar results (data not shown). After a burn-in period of 10,000 cycles, 10,000 Markov Chain Monte Carlo iterations were performed for K (number of clusters) ranging from 1–10. We used the *ad hoc* statistic ΔK (Evanno *et al.* 2005) to determine the most likely value of K and performed 100 simulations for each K.

We also used a spatial clustering method by including information on the geographical location in BAPS version 5.3 that allows a high resolution of detection, even with a low number of markers and individuals (Corander *et al.* 2008). During the clustering process, the landscape occupied by a discrete population is divided into a Voronoi tessellation (Deussen *et al.* 2000). For visual presentation of the tessellation, cells with different shades of grey representing genetically differentiated spatial groups, were calculated using the a priori assumption of dependence between neighboring cells. We fitted the model with a maximum of 20 genetic clusters and performed 50 independent runs to find the optimal partition.

An AMOVA (Excoffier *et al.* 1992) was performed to partition the genetic variation into among-spatial groups (F_{ST}), among-populations (F_{SC}), and within-populations component (F_{CT}) using the program ARLEQUIN version 3.5 (Excoffier *et al.* 2005). To obtain the significances of the variance components, we performed 5,000 random permutations. The pairwise genetic differences (F_{ST}) were calculated using the same program and were tested with 1,000 permutations at a significance level of $\alpha = 0.05$. To test for isolation by distance (Wright 1943), we correlated the matrix of geographic distances between all 24 population pairs with the matrix of pairwise F_{ST} values with the program ARLEQUIN using a Mantel test and 1,000 permutations. We also performed Mantel tests to test for isolation by distance within the two spatial groups inferred from Bayesian analyses, separately.

Genetic diversity and inbreeding

To estimate genetic diversity within all populations, we calculated the observed heterozygosity (H_o) and the expected heterozygosity (H_e) according to Nei (1978) for each population and averaged over all loci using GENALEX version 6.0 (Peakall & Smouse 2006). The mean number of alleles (N_A) per population based on the five loci was calculated with the same program. The program GENEPOP version 4.0 (Rousset 2008) was used to estimate the inbreeding coefficient (F_{IS}; Weir & Cockerham 1984) for each population across all loci. To assess the significance of the F_{IS} values for populations, departure from Hardy-Weinberg equilibrium (HWE) was evaluated with a global test at the population level for heterozygote deficit across loci. To test for linkage disequilibrium of all pairs of loci, Fisher's exact test was applied. All mentioned tests were performed using GENEPOP and were calculated with the Markov Chain algorithm (Guo & Thompson 1992) using 10,000 permutations, 20 batches, 5,000 iterations per batch and a Bonferroni correction for multiple comparisons.

To test if genetic diversity is correlated with population characteristics such as altitude or population size, we related diversity indices (H_e, N_A) and inbreeding coefficient (F_{IS}) with all measured field data (Table 1). We performed Pearson's correlation analyses with R version 2.12.1 (R Development Core Team 2010).

Genetic bottlenecks

If historic data of population size are missing, recent genetic bottlenecks (i.e. approximately within the last 12 generations) can be inferred from tests for heterozygosity excess. This allowed us to compare the expected heterozygosity (H_e) according to Nei (1973) to the heterozygosity expected at mutation-drift equilibrium (H_{eq}) using the program BOTTLENECK version 1.2 (Piry *et al.* 1999). Tests for excess of heterozygosity ($H_e > H_{eq}$) should not be confused with the above mentioned test for HWE which calculates the deficit of heterozygotes ($H_e > H_o$) when a population is in HWE. We used the Wilcoxon's test, which is the most powerful and robust test when used with a few polymorphic loci (Piry *et al.* 1999). The test was performed under the strict Stepwise Mutation Model (SMM) and the Two-Phase Model (TPM) with 95% single-step mutations and 12% variance among multiple steps (G. Luikart, University of Montana, personal communication). Significant excess of heterozygosity was tested with 1,000 permutations. Both mutation models are assumed for microsatellite evolution, but the TPM might be more appropriate for most microsatellites (Goldstein & Pollock 1997; Balloux & Lugon-Moulin 2002).

In populations that experienced a genetic bottleneck during colonisation, the occurrence of rare alleles is unlikely since the founder individuals carry only a small sample of alleles often including only the most common alleles of the source population (Nei *et al.* 1975; Slatkin 1977). For this reason, we searched for the occurrence of private alleles, i.e. rare alleles confined to only one population, and calculated the mean number of private alleles (N_P) using the program GENALEX version 6.0 (Peakall & Smouse 2006).

Results

A total of 42 unambiguously scorable and reproducible alleles ranging from 92–188 bp at five microsatellite loci were detected across all individuals. We found no evidence for linkage disequilibrium between any of the pairs of loci.

Genetic differentiation and structure

Genetic differentiation among all populations was $F_{ST} = 0.063$ (95% ci, 0.048–0.082), $G_{ST} = 0.099$ (95% ci, 0.088–0.112), and $G'_{ST} = 0.32$. The Evanno *ad hoc* statistic (see Appendix 2) for results of clustering analysis with STRUCTURE revealed two clusters of uneven size to capture the genetic structure best (Fig. 2).

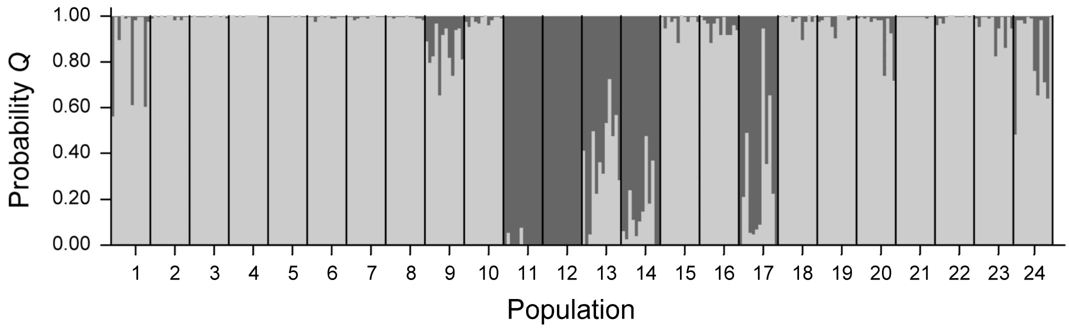


Fig. 2. Results of a cluster analysis with the program STRUCTURE (Hubisz *et al.* 2009) using microsatellite data of 24 populations of *Campanula thyrsoides* from the Schynige Platte for the most likely number of clusters $K = 2$. The different clusters are represented by different shades of grey. Individuals ($n = 288$) are grouped to populations which are aligned from west (left) to east (right). Bars indicate the assignment probability Q of individuals to be a member of one of the clusters. Shown is the simulation run with the highest likelihood for posterior distribution of data out of 100 independent runs.

For the most likely partition, the spatial clustering analysis using BAPS also revealed two groups, including the same populations (Fig. 3).

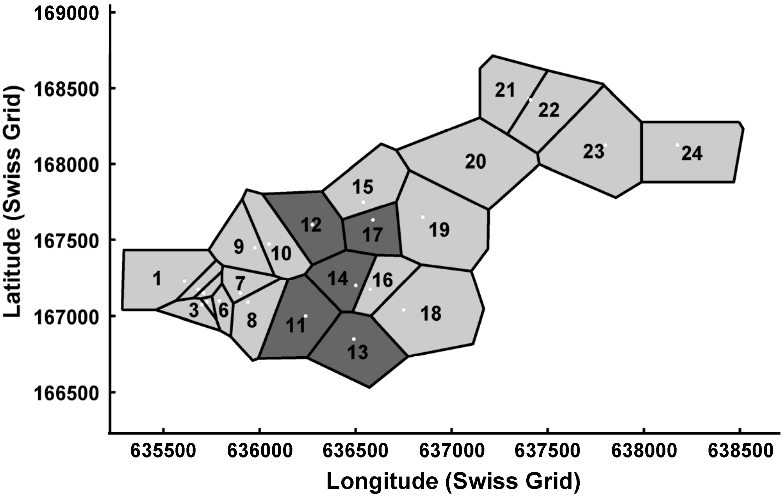


Fig. 3. Results of a spatial cluster analysis with the program BAPS (Corander *et al.* 2008) using microsatellite data of 24 populations ($n = 288$) of *Campanula thyrsoides* from the Schynige Platte. Shown is the most likely partition out of 50 independent runs with two clusters. The different clusters are represented by different shades of grey. The cells were calculated with a Voronoi tessellation (Deussen *et al.* 2000) and the center of a cell shows the population location. For a better visual presentation, populations no. 2, 4 and 5 are not numbered (but see Fig. 1 for locations).

The variance components inferred from the AMOVA were all significant with $F_{\mathrm{ST}} = 0.076$ for differentiation among the two spatial groups and $F_{\mathrm{SC}} = 0.052$ for differentiation among populations within groups (Table 2).

Table 2. Molecular variance analysis (AMOVA) of 24 populations of *Campanula thyrsoides* from the Schynige Platte

Source of variation	Sum of squares	Variance components	Percentage of variation	Fixation indices
Among groups	12.3	0.05	2.5	FST = 0.076***
Among populations within groups	88.4	0.10	5.1	FSC = 0.052***
Within populations	913.3	1.78	92.4	FCT = 0.025***
Total	1014.0	1.93		

Groups represent the two spatial genetic groups as inferred from Bayesian cluster analyses. Significance was assessed by 5,000 permutations.

***$P < 0.001$.

F_{ST} of pairwise genetic differentiation ranged from 0.001–0.191 (see Appendix 3), whereby 232 out of all 276 comparisons were significant. The pairwise geographic distances ranged from 11–2,710 m with a mean (± SD) of 999 m (± 675 m) (see Appendix 3). From 276 population pairs, three were separated by < 50 m and 13 by < 100 m. There was no evidence for an isolation by distance pattern, neither when including all 24 population pairs in the Mantel test ($r = -0.22$, $P = 0.99$), nor when testing the larger spatial group ($r = -0.25$, $P = 0.99$) or the smaller group ($r = 0.98$, $P = 0.34$) separately. The extent of pairwise genetic differentiation was irregularly scattered throughout the landscape, without any significant relationship to geographic distance (Fig. 4).

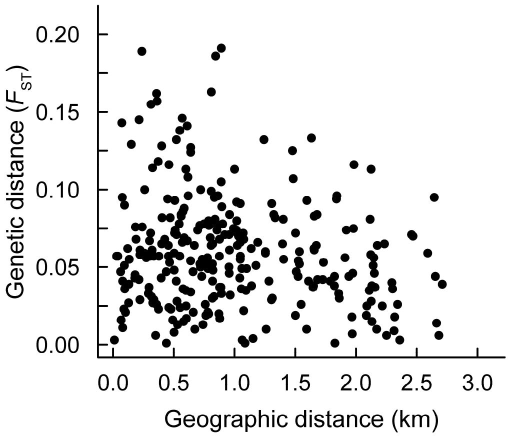


Fig. 4. Relationship of pairwise genetic differences (F_{ST}) and geographic distances of 24 populations of *Campanula thyrsoides* from the Schynige Platte. The Mantel test for correlation was not significant ($r = -0.22$, $P = 0.99$).

Genetic diversity and inbreeding

Mean observed and expected heterozygosity across all populations were $H_o = 0.75$ and $H_e = 0.71$, respectively (Table 3). Mean number of alleles across all populations was 5.0 ± 0.6 (± SD) with a range of 3.8–6.2. Mean population size was 209 and ranged from 12–780 (Table 1). Genetic diversity measured as H_e correlated negatively with plant density ($r = -0.49$, $P < 0.05$). Neither genetic diversity measured as H_e or N_A, nor inbreeding measured as F_{IS} correlated with any of the tested variables (results not shown).

Table 3. Genetic diversity indices, occurrence of private alleles and inbreeding in 24 populations of *Campanula thyrsoides* from the Schynige Platte

Population	H_o	H_e	N_A	N_P	F_{IS}	Population	H_o	H_e	N_A	N_P	F_{IS}
1	0.839	0.741	5.4	0.0	-0.140	13	0.799	0.778	6.2	0.0	-0.034
2	0.833	0.757	6.0	0.0	-0.106	14	0.751	0.747	5.6	0.0	-0.006
3	**0.824**	**0.710**	**4.4**	**0.0**	**-0.161**	15	0.743	0.664	4.0	0.0	-0.131
4	0.708	0.662	4.8	0.0	-0.071	16	0.739	0.712	5.0	0.0	-0.043
5	0.700	0.686	5.6	0.0	-0.001	17	0.579	0.704	5.0	0.4	0.195*
6	0.822	0.724	4.4	0.0	-0.147	18	0.677	0.733	5.4	0.0	0.082
7	0.677	0.672	5.0	0.0	-0.010	19	0.717	0.735	5.2	0.2	0.023
8	0.608	0.576	4.6	0.0	-0.056	20	0.830	0.678	4.6	0.0	-0.246
9	0.762	0.689	5.0	0.0	-0.112	**21**	**0.697**	**0.681**	**3.8**	**0.0**	**-0.037**
10	0.793	0.772	5.6	0.0	-0.029	**22**	**0.783**	**0.717**	**4.2**	**0.0**	**-0.096**
11	**0.854**	**0.780**	**5.4**	**0.0**	**-0.107**	23	0.707	0.732	4.4	0.0	0.031
12	0.777	0.761	5.8	0.0	-0.024	24	0.684	0.723	5.2	0.0	0.059
						Mean	0.746	0.714	5.0	0.03	-0.049
						SD	0.073	0.046	0.6	0.09	0.092

Data from 288 individuals calculated across all 5 microsatellite markers. Asteriks behind F_{IS} values show significant deviation from Hardy-Weinberg equilibrium tested with a global test for heterozygote deficit. Bold values indicate bottlenecked populations (see Table 4). H_o, observed heterozygosity (Nei 1978); H_e, expected heterozygosity (Nei 1978); N_A, mean number of alleles per locus; $N_{P,}$ mean number of private alleles; F_{IS}, inbreeding coefficient (Weir & Cockerham 1984). $*P < 0.05$.

The mean inbreeding coefficient F_{IS} was -0.049 (Table 3). A positive F_{IS} value (0.195) indicated inbreeding in one population (no. 17) and the same population showed significant departure from HWE. Since no locus showed constant departures in HWE across all populations, the presence of undetected null alleles is unlikely (Chapuis & Estoup 2007).

Evidence for genetic bottlenecks

We observed a significant excess of heterozygosity in four out of 24 populations based on the Wilcoxon's test under both mutation models, indicating genetic bottlenecks in these populations (Table 4). Private alleles were detected within only two populations (no. 17 and 19) and the mean number of private alleles over all populations was low (0.03), suggesting genetic bottlenecks during colonisation (Table 3).

Table 4. Test for recent genetic bottlenecks in 24 populations of *Campanula thyrsoides* from the Schynige Platte

Population	SMM	TPM	Population	SMM	TPM
1	0.31	0.31	13	0.92	0.89
2	0.50	0.50	14	0.89	0.59
3	0.02*	0.02*	15	0.11	0.08
4	0.69	0.69	16	0.41	0.41
5	0.98	0.98	17	0.59	0.31
6	0.41	0.31	18	0.41	0.41
7	0.96	0.92	19	0.69	0.50
8	0.89	0.69	20	0.89	0.89
9	0.92	0.89	21	0.02*	0.02*
10	0.31	0.31	22	0.02*	0.03*
11	0.03*	0.02*	23	0.08	0.08
12	0.59	0.59	24	0.41	0.41

Shown are P values from the Wilcoxon's test that was performed under the Stepwise Mutation Model and the Two-Phase Model assumed for microsatellite evolution (Piry *et al.* 1999). Significant excess of heterozygosity indicates a genetic bottleneck and was assessed by 1,000 permutations. SMM, Stepwise Mutation Model; TPM, Two-Phase Model. $^*P < 0.05$.

Discussion

High genetic differentiation in a small area

Despite the small area of our study region on the Schynige Platte (10 km^2), we observed a remarkably high genetic differentiation among the 24 populations of *C. thyrsoides* ($G'_{\mathrm{ST}} = 0.32$). This is particularly true considering population density is high and that the minimal distance between two populations is, on average, only 214 meters (see Appendix 3). Differentiation among the 24 populations on this mountain plateau is astonishingly high when compared with the values observed at larger geographical scales in this species, with a G'_{ST} of 0.43 for the Central Swiss

Alps and with a G'_{ST} of 0.68 for the entire Alpine range (J. Stöcklin, University of Basel, unpublished data; Table 5).

Table 5. Comparison of genetic differentiation among 24 populations of *Campanua thyrsoides* from the Schynige Platte compared with findings at larger spatial scales in the Alps

	Area (km^2)	*n*	F_{ST}	G_{ST}	G'_{ST}
Schynige Platte	10	24	0.06	0.10	0.32
Central Swiss Alps	18 000	17	0.08	0.10	0.43
European Alps	190 000	51	0.16	0.18	0.68

Results from Central Swiss Alps and European Alps are from studies using the same species and marker type (J. Stöcklin, University of Basel, unpublished data). n; number of populations. F_{ST}, differentiation index (Weir & Cockerham 1984); G_{ST}, differentiation index (Nei 1973); G'_{ST}, standardized G_{ST} (Hedrick 2005).

Our results from *C. thyrsoides* are also outstanding when compared with one of the few Alpine studies investigating molecular differentiation in a similarly small region in the Alps; population differentiation of *Eryngium alpinum* in a 12 km long valley was $F_{ST} = 0.01$ (Gaudeul & Till-Bottraud 2008), much lower than $F_{ST} = 0.06$ in our study.

The high differentiation of *C. thyrsoides* on the plateau is a clear indication of a generally low gene flow and a highly restricted seed dispersal ability of the species as has been previously assumed (Kuss *et al.* 2007). Furthermore, the low admixture among the two spatial genetic groups inferred from the Bayesian cluster analyses, as well as the detection of genetic bottlenecks in several populations (Table 4), support the hypothesis of a weak gene flow among populations.

Spatial genetic structure

The Bayesian structuring analyses revealed an unexpected splitting of the populations into two spatial genetic groups (Figs 2 and 3). A smaller group of five populations (including the one from the Botanical Garden) was bordered on both sides by a much larger group, including 19 of the other populations on the mountain plateau. The AMOVA-derived differentiation among the two spatial groups is significant and relatively high ($F_{ST} = 0.08$; Table 2) compared with the differentiation of populations within the two groups ($F_{SC} = 0.05$). An explanation for their spatial arrangement could be that either pollen or seed dispersal from plants in the Botanical Garden (population no. 11) has affected populations in the vicinity. Seed material of *C. thyrsoides* was introduced into the Botanical Garden on the Schynige Platte in the 1950s (O. Hegg, Botanical Garden Schynige Platte, personal observation). Unfortunately, the origin of this seed material is not known, but according to our inquiries, seeds were introduced from outside the Schynige Platte, though not from outside Switzerland. Thus, plants from a distinct gene pool introduced more than a half century ago may have subsequently transmitted foreign alleles into the indigenous gene pool of this region.

Pairwise genetic differentiation

A strongly irregular structure of pairwise genetic differentiation was inferred from *F*-Statistics, meaning that even closely-located population pairs of *C. thyrsoides* on the Schynige Platte showed a large range of differentiation values (Fig. 4). The distance-independent pattern of differentiation contrasts observations at a larger Alpine-wide scale in this species (Ægisdóttir *et al.* 2009). This suggests that at the local scale of a single mountain plateau other processes are important for shaping the genetic structure in Alpine plants than at regional scales. Significant isolation by distance is lacking on the Schynige Platte and the spatial genetic structure indicates non-equilibrium processes of genetic drift and gene flow, with random drift probably more influential than gene flow (see case III in Hutchison & Templeton 1999). Drift might be particularly pronounced on the Schynige Platte due to the spatial isolation of populations related to the complex topography and the distinct slope and exposition of their habitats (Fig. 1; Table 1).

We assume that irregular and randomly occurring gene flow among populations of *C. thyrsoides* on the Schynige Platte is contributing largely to the pattern of genetic diversity and differentiation within and among populations. The random nature of long-distance seed dispersal, i.e. the lack of a predominant dispersal direction (Cain *et al.* 2000; He *et al.* 2010), may have led to occasional migration among populations in this fragmented landscape. During colonisation, founder effects may have been frequent on the mountain plateau, since only a few seeds from a single plant may have reached suitable habitats located far away from the source. Such founder effects are indicated by genetic bottlenecks in several populations, including the one situated within the Botanical Garden (population no. 11; Table 4). Unfortunately, detailed knowledge on the occurrence and frequency of long-distance seed dispersal in *C. thyrsoides* is still missing. It might however well be that secondary dispersal over snow or ice is a more efficient mechanism for long-distance dispersal of seeds than primary wind dispersal (Morton & Hogg 1989).

High genetic diversity

The average genetic diversity within populations of *C. thyrsoides* on the Schynige Platte was higher ($H_e = 0.71$) than the reported average ($H_e = 0.61$) of other microsatellite studies (reviewed in Nybom 2004), but is well in line with the observed high genetic diversity in this species in other populations in the Swiss Alps and Jura mountains (Ægisdóttir e*t al.* 2009). On the Schynige Platte even small populations including 30 or less individuals exhibit high genetic diversity (Tables 1 and 3). As already concluded earlier (Kuss *et al.* 2008a, b), the predominantly outbreeding system in *C. thyrsoides* may be responsible for the high genetic diversity in this monocarpic plant.

Since we found a negative correlation of genetic diversity with the plant density of a population, it could be assumed that this relationship is due to shorter pollen dispersal distances, thereby a higher bi-parental inbreeding in denser populations (Schmitt 1983). As could be expected (Ouborg & van Treuren 1994; Gaudeul *et al.* 2000), we found neither a significant relationship of genetic diversity with population size nor between inbreeding and population size. Thus, we conclude that the high generation overlap together with the longevity of individuals in this perennial species (Kuss *et al.* 2008b) may have counteracted low genetic diversity and inbreeding depression in small populations.

Low inbreeding despite genetic bottlenecks

The average inbreeding was close to zero ($F_{\mathrm{IS}} = -0.05$) indicating random mating (Table 3). Only in one population (no. 17) out of 24 populations of *C. thyrsoides*, inbreeding was indicated by our molecular data. This may be due to sister-mating and bi-parental inbreeding (Ægisdóttir *et al.* 2007a). Despite little evidence for general inbreeding and the already mentioned high genetic diversity in most populations, we found evidence of genetic bottlenecks in four populations (Table 4). The presence of bottlenecks is further supported by the absence of private alleles in these populations (Table 3). Most likely the detected bottlenecks mirror founder effects during colonisation, i.e. founding of populations by only a few individuals, since the bottlenecked populations are currently large and three out of four have high numbers of flowering individuals (Table 1). Because bottlenecked populations were not inbred (Table 3), it suggests that negative effects of small founding populations (Ellstrand & Elam 1993) were either low or have been overcome, for example, through repeated bottlenecks which can lead to a reduction of the genetic load (Barrett & Charlesworth 1991). To sum up, we suggest that the high generation overlap and the longevity of individuals or repeated bottlenecks have prevented or subsequently overcome strong inbreeding in this species.

Conclusions

At a small spatial scale, we observed an unexpectedly high genetic differentiation and diversity, and found little evidence for inbreeding in the monocarpic Alpine plant *C. thyrsoides* on a mountain plateau in the Swiss Alps. In contrast to findings in this species at larger Alpine-wide scales, isolation by distance is less important for shaping the spatial genetic structure on this small plateau. Moreover, in the context of a highly structured landscape, our results suggest that drift and random dispersal events, including occasional migration and colonisation associated with genetic bottlenecks, are among the most relevant factors for the genetic structure in *C. thyrsoides*. Finally, results indicate that, despite highly restricted seed dispersal, occasional gene flow is sufficient to ensure genetic diversity and to overcome negative effects of bottlenecks in this species. This is surely a large advantage for the population persistence of this rare bell flower when its habitats in the Alps are shrinking in times of global warming and land use changes.

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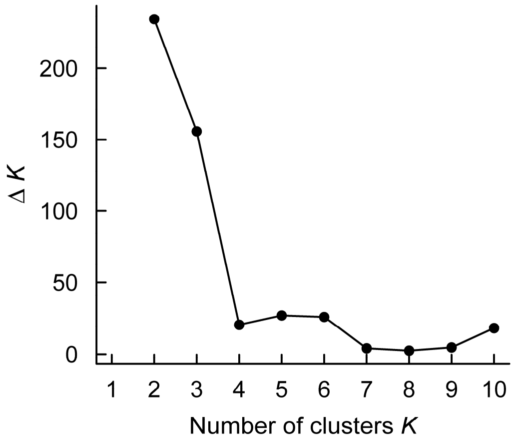
Appendix

Appendix 1. Locus name, repeat motif, GenBank accession number and primer sequence for five *Campanula thyrsoides* microsatellite loci

Locus	Repeat	GenBank accession number	Primer sequence (5'–3')
Campthy 1	$(CA)_{16}$	EF371506	F: CTGCTAGGCTATGCGAGTGTTC R: TCTGAATTTGTTGAGAATCTTTTTG
Campthy 3	$(CA)_{13}$	EF371507	F: AAAGTTTGATTCCAAGGTGCTC R: AAAATAATTCCAGGGACGGAGT
Campthy 5	$(GT)_{20}$	EF371508	F: CCAGCGACGCTTTAGTTATTGT R: CAAATATAAAGGGGAAGTTACTTATCA
Campthy 6	$(CA)_{17}$	EF371509	F: ACAACCTCGAACCAATTTTCAG R: CAATTGGGGTCTAACCATTCAC
Campthy 9	$(CA)_{24}$	EF371510	F: AATGTCCATGGTGTGGTGAAC R: CCATTCAAAGCCGCAGTATTAG

For details see Ægisdóttir *et al.* (2007b).



Appendix 2. *Ad hoc* statistic (Evanno *et al.* 2005) of a cluster analysis with the program STRUCTURE (Hubisz *et al.* 2009) using microsatellite data of 24 populations of *Campanula thyrsoides* from the Schynige Platte. The highest value of Δ*K* at the true number of clusters (*K* = 2) is shown.

Appendix 3. Pairwise genetic and geographic distances of 24 populations of *Campanula thyrsoides* from the mountain plateau of the Schynige Platte in the Swiss Alps

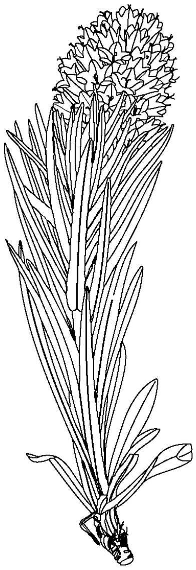
Pop	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1		0.089	0.179	0.128	0.181	0.222	0.300	0.358	0.425	0.503	0.669	0.759	0.956	0.888	1.063	0.964	1.056	1.152	1.306	1.679	2.151	2.156	2.360	2.710
2	0.023		0.102	0.039	0.096	0.133	0.221	0.273	0.403	0.476	0.585	0.730	0.871	0.818	1.033	0.892	1.015	1.075	1.260	1.646	2.125	2.130	2.318	2.663
3	0.043	0.021		0.076	0.029	0.093	0.213	0.240	0.465	0.531	0.544	0.777	0.819	0.807	1.076	0.878	1.047	1.048	1.283	1.683	2.170	2.175	2.342	2.682
4	0.027	0.057	0.095		0.062	0.094	0.189	0.237	0.400	0.470	0.549	0.721	0.834	0.789	1.022	0.863	1.000	1.043	1.242	1.633	2.116	2.121	2.301	2.644
5	0.045	0.036	0.057	0.047		0.066	0.184	0.214	0.438	0.503	0.521	0.749	0.800	0.781	1.047	0.852	1.018	1.023	1.254	1.654	2.141	2.146	2.313	2.653
6	0.061	0.055	0.051	0.090	0.016		0.120	0.150	0.396	0.456	0.460	0.696	0.741	0.715	0.991	0.786	0.958	0.959	1.191	1.595	2.085	2.089	2.251	2.590
7	0.067	0.058	0.042	0.068	0.076	0.062		0.072	0.309	0.358	0.371	0.585	0.660	0.600	0.876	0.673	0.839	0.855	1.071	1.477	1.969	1.973	2.131	2.469
8	0.162	0.056	0.076	0.189	0.145	0.129	0.143		0.362	0.400	0.312	0.610	0.599	0.569	0.891	0.639	0.844	0.809	1.066	1.483	1.979	1.983	2.124	2.457
9	0.068	0.082	0.053	0.128	0.047	0.043	0.072	0.157		0.079	0.522	0.335	0.790	0.580	0.638	0.658	0.639	0.875	0.895	1.254	1.726	1.732	1.941	2.295
10	0.059	0.023	0.013	0.082	0.048	0.057	0.026	0.065	0.041		0.511	0.257	0.764	0.526	0.561	0.603	0.560	0.822	0.817	1.176	1.650	1.656	1.862	2.216
11	0.064	0.069	0.078	0.138	0.132	0.116	0.118	0.155	0.071	0.074		0.601	0.291	0.327	0.808	0.377	0.720	0.510	0.890	1.334	1.839	1.842	1.920	2.234
12	0.026	0.044	0.056	0.100	0.078	0.074	0.088	0.141	0.057	0.059	0.025		0.780	0.459	0.304	0.520	0.316	0.734	0.575	0.919	1.395	1.401	1.609	1.966
13	0.046	0.020	0.041	0.095	0.069	0.053	0.021	0.113	0.052	0.019	0.033	0.021		0.350	0.902	0.336	0.787	0.321	0.877	1.323	1.822	1.823	1.826	2.109
14	0.037	0.052	0.064	0.077	0.049	0.052	0.035	0.146	0.015	0.028	0.027	0.024	0.006		0.552	0.079	0.439	0.296	0.570	1.017	1.523	1.525	1.593	1.909
15	0.003	0.070	0.068	0.072	0.066	0.075	0.068	0.191	0.054	0.083	0.099	0.057	0.078	0.024		0.576	0.130	0.740	0.325	0.616	1.094	1.099	1.311	1.673
16	0.032	0.033	0.017	0.096	0.078	0.080	0.010	0.124	0.047	0.017	0.057	0.041	0.029	0.011	0.036		0.455	0.221	0.549	0.998	1.501	1.502	1.548	1.857
17	0.036	0.074	0.091	0.113	0.080	0.061	0.081	0.186	0.034	0.084	0.050	0.031	0.020	0.001	0.039	0.064		0.611	0.260	0.639	1.137	1.140	1.304	1.656
18	0.004	0.022	0.057	0.043	0.064	0.084	0.074	0.163	0.057	0.048	0.072	0.055	0.061	0.037	0.013	0.029	0.067		0.618	1.047	1.534	1.534	1.508	1.788
19	0.091	0.010	0.041	0.132	0.060	0.066	0.072	0.069	0.089	0.030	0.105	0.086	0.037	0.066	0.114	0.057	0.100	0.096		0.449	0.954	0.955	1.060	1.404
20	0.084	0.037	0.042	0.133	0.062	0.093	0.125	0.107	0.059	0.051	0.082	0.071	0.084	0.092	0.108	0.074	0.127	0.055	0.094		0.506	0.508	0.714	1.086
21	0.046	0.028	0.010	0.081	0.051	0.019	0.007	0.075	0.042	0.038	0.094	0.065	0.038	0.044	0.035	0.019	0.062	0.060	0.050	0.072		0.011	0.502	0.831
22	0.037	0.038	0.064	0.058	0.056	0.025	0.046	0.116	0.053	0.061	0.096	0.081	0.044	0.054	0.049	0.072	0.038	0.052	0.071	0.093	0.003		0.491	0.820
23	0.003	0.018	0.026	0.036	0.009	0.006	0.015	0.113	0.044	0.030	0.074	0.041	0.001	0.010	0.030	0.026	0.029	0.047	0.049	0.077	0.008	0.016		0.374
24	0.039	0.014	0.006	0.095	0.044	0.059	0.070	0.071	0.040	0.025	0.065	0.018	0.035	0.056	0.064	0.033	0.083	0.041	0.055	0.001	0.031	0.065	0.023	

Genetic distances (F_{ST}) are shown below diagonal and geographic distances (in km) are shown above diagonal.

Chapter 5

Monocarpic perenniality of *Campanula thyrsoides* results in high population differentiation despite high pollen flow

J.F. Scheepens, **Eva S. Frei**, Georg F.J. Armbruster &
Jürg Stöcklin



Summary

Populations of the bellflower *Campanula thyrsoides* L. harbour high genetic diversity but are simultaneously strongly differentiated even at small spatial scales. The monocarpic perennial nature of *C. thyrsoides* could explain this seeming contradiction as it theoretically limits genetic exchange among populations in a temporal way: a great amount of genetic diversity in a population is stored in non-flowering individuals, since each year less than 10% of individuals flower and potentially exchange only a small proportion of the total genetic material among populations. Based on this reasoning, we hypothesised that considerable gene flow, mainly by pollen, is present among populations. We therefore performed a paternity analysis using six microsatellite loci to estimate the amount of pollen flow into a single population situated on a subalpine mountain plateau in Central Switzerland. This plateau harboured 24 populations, occupying an area of < 5 km^2. Analysis of 331 offspring from 22 mother plants indicated a minimum of 7.6% gene flow into the study population. All 25 immigrants originated from neighbouring populations within a radius of 1,000 m. The pollination distances to mother plants were affected by their spatial isolation in the population, but variability in male mating success was not related to degree of isolation of father plants. Additional fluorescent pollen experiments showed larger pollination distances than paternity analysis results, possibly due to different methods or environmental variability. The detected pollen flow is substantial but apparently not sufficient to diminish the high genetic differentiation among populations, likely as a consequence of the species' monocarpic perenniality.

Key-words: European Alps, fluorescent pollen analogues, gene flow, male mating success, paternity analysis, pollination distance

Introduction

Gene flow is the change in gene frequencies in a population due to movement of external gametes or individuals into that population (Slatkin 1987). In flowering plants, gene flow occurs through the establishment of individuals bearing new genes that entered the population through either seed or pollen dispersal. Various factors can affect patterns of gene flow, such as the spatial positioning of populations (Heywood 1991), landscape elements obstructing or promoting dispersal (Manel *et al.* 2003), pollinator abundance and activity (Utelli & Roy 2000), the breeding system of the species (Loveless & Hamrick 1984; Hamrick & Godt 1996) and adaptations of seeds or pollen to efficient dispersal (Van der Pijl 1982; Loveless & Hamrick 1984).

Gene flow is an important aspect of the biology of a species as it affects the genetic diversity of its populations and thereby influences the course of evolution. It is generally believed that gene flow prevents evolution as it constrains adaptation to local conditions, but it may as well promote evolution by introducing novel, advantageous genes into a population (Slatkin 1987). Gene flow also counteracts drift and may prevent genetic erosion or inbreeding effects in small populations (Young *et al.* 1996; Conner & Hartl 2004).

Dispersal of seeds and pollen can be estimated using indirect or direct methods. Indirect methods use genetic markers to assess long-term dispersal patterns. Here, migration rates are deduced from estimates of molecular among-population differentiation, but this method is highly criticised as natural conditions violate important theoretical assumptions (Whitlock & McCauley 1999). In order to assess contemporary dispersal patterns, direct methods of dispersal involve observation of pollen or seed dispersal in the landscape, either through tracking or trapping propagules (Bullock *et al.* 2006). Pollen dispersal can also be investigated quasi-directly using fluorescent pollen analogues applied to a flowering source individual (Stockhouse 1976; Waser 1988; Van Rossum *et al.* 2011). However, successful pollination can deviate strongly from observed pollen dispersal or vector movement due to factors such as self-incompatibility (Ægisdóttir *et al.* 2007a;

Llaurens *et al.* 2008) and heterostyly (Kohn & Barrett 1992). An alternative method is to reconstruct the effective gene flow distances between parents and their offspring as inferred from molecular markers in parentage analysis (Streiff *et al.* 1999; Ashley 2010).

In this study, we investigated pollen dispersal in a widespread monocarpic perennial from the European Alps, *Campanula thyrsoides*, by means of paternity analysis and fluorescent pollen analogues. Populations of this species, which are usually isolated from each other, have been shown to exhibit high levels of within-population genetic diversity ($H_E = 0.76$) and a low inbreeding coefficient ($F_{IS} = 0.022$), probably due to the species' strong but incomplete self-incompatibility (Ægisdóttir *et al.* 2007a). The species' among-population differentiation is substantial at various scales (European Alps: $G'_{ST} = 0.68$; Central Swiss Alps phylogeographic region: $G'_{ST} = 0.43$; Schynige Platte local scale: $G'_{ST} = 0.32$; Kuss *et al.* 2011; Frei *et al.*, in review) and shows significant isolation by distance (Kuss *et al.* 2008a; Ægisdóttir *et al.* 2009).

The observed high within-population genetic diversity seems to contradict the substantial among-population differentiation of *C. thyrsoides*. On the one hand, the substantial among-population differentiation suggests restricted gene flow, in line with the strong isolation of populations, low population sizes and low seed dispersal capacity, allowing for genetic drift to occur. On the other hand, the high genetic diversity within populations suggests considerable gene flow, which prevents genes from going extinct (Conner & Hartl 2004). The monocarpic life cycle of *C. thyrsoides* could be the key to understanding this putative contradiction, as monocarpic perenniality theoretically limits genetic exchange among populations in a temporal – not spatial – way (Vitalis *et al.* 2004). The age to flowering is on average 10 years (Kuss *et al.* 2008b), which means that, due to mortality before flowering, less than 10% of the population flowers annually. Therefore, even if pollen flow is extremely efficient among populations, only a limited set of alleles would be exchanged, with about 90% of genetic material being immobilised in non-flowering individuals. Thus, the high genetic diversity in populations of this species could be maintained by efficiently spreading genes among populations and storing them in vegetative offspring. Simultaneously, the temporally limited genetic exchange could explain the observed among-population differentiation (Vitalis *et al.* 2004). In line with these theoretical considerations on the population-genetic effects of monocarpic perenniality, we hypothesise that the proportion of pollen dispersal over long distances is considerable in *Campanula thyrsoides*.

In summary, we expect considerable pollen dispersal among populations of *Campanula thyrsoides*, as this explains how populations retain high genetic diversity despite their spatial isolation, low population sizes and low seed dispersal capacities. We therefore aim to reconstruct pollen movement within and into a single population on a Swiss subalpine mountain plateau harbouring 24 populations. We apply two different methods: i) paternal assignment using microsatellite data (Streiff *et al.* 1999; Oddou-Muratorio *et al.* 2005; Ashley 2010); ii) direct observations of pollination using fluorescent pollen analogues (Stockhouse 1976; Waser 1988; Van Rossum *et al.* 2011). Since seed dispersal is highly limited in *C. thyrsoides* (Kuss *et al.* 2007), investigating pollen flow by analysing seeds sampled from mother plants is likely to give a realistic picture of overall gene flow (Bacles & Ennos 2008). Besides estimating pollen flow into the population from the paternal assignment analysis, we applied dispersal models to the data (Bullock *et al.* 2006; Pluess *et al.* 2009) in order to infer the relative amount of dispersal into the population. In particular we ask the following questions: 1) What fraction of the pollen contributions comes from outside the population?; 2) Where do immigrants originate?; 3) Has the spatial location of the mother and father plants within the population any influence on pollination distances and paternal success?; 4) How do the paternal analysis and fluorescent pollen estimates of pollen movement differ?

Materials and methods

Study species

Campanula thyrsoides L. (Campanulaceae) is a rosette-forming monocarpic perennial occurring in the European Alps, Jura Mts and the Dinaric Arc (Aeschimann *et al.* 2005; Kuss *et al.* 2007). The species occurs in sub-alpine and alpine grasslands on carbonate-bearing soils, typically between 1,600–2,200 m a.s.l.

(Kuss *et al.* 2007). Initiation of flowering is dependent on the rosette size. Based on integral projection models as well as herb chronology, Kuss *et al.* (2008b) estimated the average flowering age at about 10 years with a broad range of 3–16 years (Kuss *et al.* 2007). The inflorescence bears on average 50 densely-packed, bell-shaped, protandrous flowers which open within a few days (Scheepens *et al.* 2011). The species has a gametophytic self-incompatibility system, but is able to mate with half-sibs (Ægisdóttir *et al.* 2007a). Bumblebees are the main pollinators (Ægisdóttir *et al.* 2009). Previous direct measurements of pollen dispersal revealed restricted within-population dispersal by insects (mean ± SD = 4.85 ± 7.1 m; max = 39 m; Ægisdóttir *et al.* 2009). Seeds, which lack morphological adaptations for dispersal (Kuss *et al.* 2007, 2008a), are shaken out of the capsules by wind, rain or animal activity. Based on a wind dispersal model, 99.99% of seeds would be dispersed within 10 m to the mother plant (Kuss *et al.* 2007). Populations of this diploid (2n = 34, see references in Ægisdóttir *et al.* 2009) are naturally isolated, usually by large distances (Kuss *et al.* 2007).

Study system

The Schynige Platte is a subalpine, south-east-facing mountain plateau (ca 4.4 km^2) with calcareous bedrock located at 1,750–2,100 m a.s.l. in the northern Swiss Alps (46°39’26”N; 7°55’18”E). Average annual precipitation is 1,716 mm and annual minimum, mean and maximum temperatures are –8.5, 2.0 and 13.8°C, respectively (based on monthly averages, WorldClim data, Hijmans *et al.* 2005). A total of 24 populations of *Campanula thyrsoides* is located in this area (Fig. 1). These populations differ in their area (60–6,500 m^2), isolation (nearest population 11–449 m) and population size (estimates from the year 2006): 12–700 rosettes and flowering individuals.

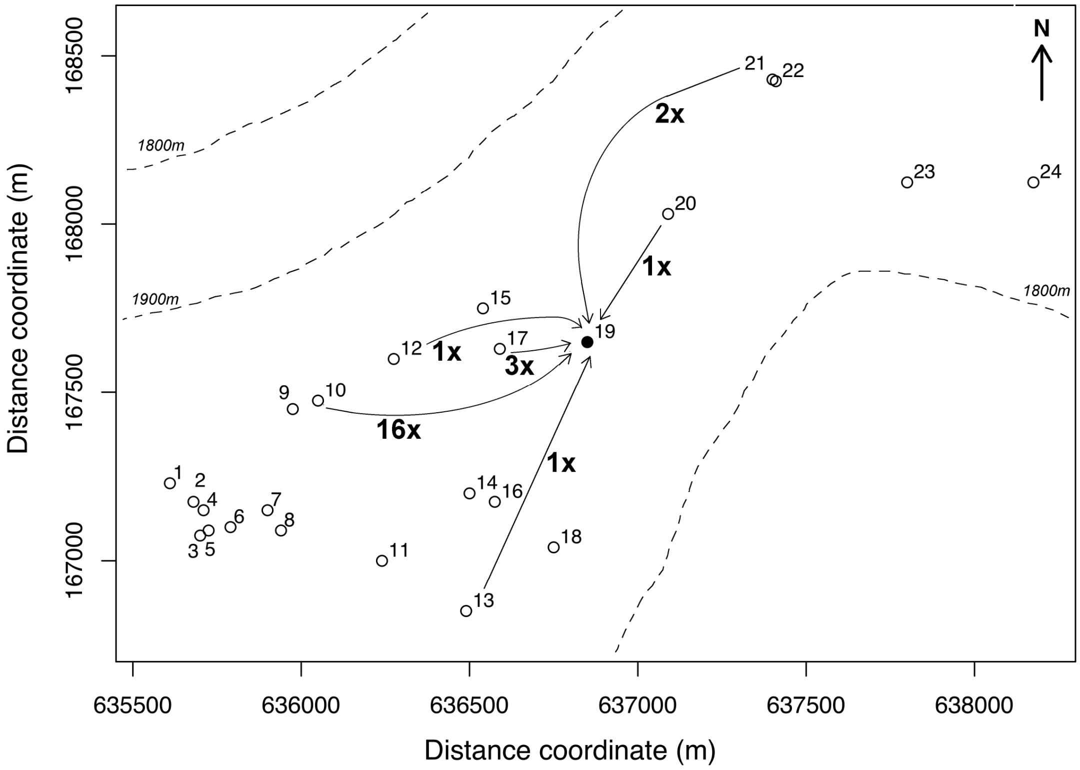


Fig. 1. Population locations of *Campanula thyrsoides* on the Schynige Platte. Arrows with numbers indicate events of gene flow into the study population (filled circle) based on the rare-allele method using the programs CERVUS (Kalinowksi *et al.* 2007) and GENECLASS2 (Piry *et al.* 2004; see Materials and methods and Table 4). Coordinates according to Swiss grid. Isolines are schematic.

Substantial population differentiation was found across spatial levels, ranging from the scale of the European Alps ($G'_{ST} = 0.68$), the Western Alpine phylogeographic region ($G'_{ST} = 0.53$), to the landscape level of the mountain plateau of Schynige Platte ($G'_{ST} = 0.32$; Frei *et al.*, in review). The study population (no. 19; Fig. 1) lies east of the centre of gravity of the population distribution on the Schynige Platte at an elevation of 1,950 m a.s.l. with a southeastern exposition and an estimated inclination of 30°. Its three neighbouring populations are located 261, 326 and 449 m away. The total occupying area is ca 6,500 m^2, the vegetation cover is 95%, the number of flowering individuals ranged from 22–105 over five years (2005–2009) and the effective population size based on the harmonic mean of yearly varying flowering individuals is $N_e = 37.6$ (Conner & Hartl 2004). However, Vitalis *et al.* (2004) theorise that the effective population size of monocarpic species is always larger than the number of flowering individuals in a given year. Based on five microsatellite loci investigated in leaf samples from flowering individuals in 2006 (Frei *et al.*, in review), H_E of the study population was estimated at 0.735, $H_O = 0.717$ and $F_{IS} = 0.023$. This F_{IS} was non-significant (based on a test for heterozygote deficit on all 24 populations across all loci; Frei *et al.*, in review), and the population did not show signs of recent bottlenecks.

Paternity analysis

Sampling design

On 14 August 2007, 22 individuals flowered in the study population and leaf tissue of all flowering individuals was sampled and stored in 2 mL Eppendorf tubes containing silica gel. On 28 October 2007, the location of all previously flowering individuals was recorded and ripe seeds were sampled from these mother plants. We grew offspring in a greenhouse from randomly selected seeds, which we assumed to be derived from separate pollinator visits. This assumption was based on the observation that inflorescences bear on average 50 flowers (Scheepens *et al.* 2011) each with ca 150 seeds (Kuss *et al.* 2007) and individual pollinators usually pollinate only one to a few flowers (J.F. Scheepens, personal observation). We sampled the offspring for leaf tissue, which was stored in 2 mL Eppendorf tubes containing silica gel. We successfully grew offspring from 20 out of 22 mother plants, totalling 338 and ranging from 2–38 offspring per mother plant (median = 15).

DNA extraction and PCR amplification

To extract total DNA from leaf material of 360 samples (22 mother plants and 338 offspring), silica-dried leaf material was milled (Retsch MM300; Retsch, Haan, Germany) and a DNeasy Plant Mini Kit (Qiagen, Hombrechtikon, Switzerland) was used to extract DNA, following a slightly modified manufacturer's protocol, which added a proteinase K treatment after the prescribed RNase A addition.

We screened six polymorphic microsatellites: Campthy 1, Campthy 3, Campthy 5, Campthy 6, Campthy 9 and Campthy 15 (Ægisdóttir *et al.* 2007b). Linkage disequilibrium has not been detected for these loci (Ægisdóttir *et al.* 2007b). Polymerase chain reactions (PCR) were performed on an Eppendorf

MasterCycler Gradient (Vaudaux-Eppendorf, Schönenbuch, Switzerland) in 10 µL reaction volumes of which 3 µL total DNA solution (30–100 ng), 1 µL of 10× PCR buffer, 0.125 µM each of forward and reverse primer, 150 µM dNTP and 1 U HotstarTaq polymerase (Qiagen, Hombrechtikon, Switzerland). After a denaturation step of 15 min at 95°C, 30 cycles of 30 s annealing at primer-specific temperatures (Campthy 1, 3, 5, 6, 9: 56°C; Campthy 15: 60°C) followed by 30 s at 70°C and 30 s at 95°C were performed, with a final 10 min extension at 70°C. Horizontal gel electrophoresis of PCR products was performed using Spreadex® gels with a resolution of 2 basepairs in a SEA-2000TM submerged gel electrophoresis system (Elchrom Scientific, Cham, Switzerland). Ethidium bromide-stained (1mg/mL) gels were photographed under UV light.

Data scoring

Scoring of bands was performed by repeated manual verification of all samples without knowing the sample relationships. Any samples with unclear genotype patterns were repeated. Three randomly chosen blind samples (ca 1%) were repeated and the error rate established as allelic differences between these duplicates at 7.1%. A previous estimate of the error rate, using five similar markers and 39 repeated samples, was 6.1% (Kuss *et al.* 2011). Thereafter, mother and offspring genotypes were compared to check for consistent heritability of the maternal alleles to the offspring. Based on this analysis, specific alleles were binned to remove part of the scoring and genotyping errors (Appendix 1; Bacles & Ennos 2008). This binning was based on the two criteria that (1) binning would solve ambiguous allele assignment and that (2) binning would solve mother-offspring inconsistencies that occurred regularly in the dataset. We assumed that the range of mother-offspring mismatches covered the overall scoring and genotyping error in the dataset, so that solving mismatches would also positively affect the assignment to fathers. Any remaining mother-offspring inconsistencies were solved by replacing one of the homozygote offspring allele with a missing allele. In fact, such inconsistencies could be due to null alleles, for which the replacement by missing alleles is appropriate (Wagner *et al.* 2006; Bacles & Ennos 2008). In the forthcoming analysis, we compared the performance of the original (RAW) and the binned (BIN) dataset.

Molecular data analysis

We used the program CERVUS 3.0.3 (Kalinowksi *et al.* 2007) for paternal analysis. CERVUS performs assignment of offspring to one or both parents based on maximum likelihood and performs an offspring simulation run on parental genotypic data to establish threshold values of confidence in the assigned offspring. We ran an analysis for the RAW and BIN dataset separately using the following parameters: Mean number of candidate fathers was 22, selfing is possible, and the proportion of potential fathers genotyped was 1.00 as all flowering individuals in the population were screened. The applied genotyping error rates were based on mother-offspring inconsistencies for the RAW and BIN data sets. For the BIN dataset, the error rate was taken after binning but before solving remaining parent-offspring consistencies by replacement with missing alleles. We used the same values for the likelihood error rate and the genotyping error rate. The number of mismatching seed genotypes is given (N_{mismatch}) per number of tested individuals ($N_{\mathrm{comparison}}$) (Table 1; Bacles & Ennos 2008). Inbreeding can be simulated in CERVUS but since no inbreeding was detected in the study population (Frei *et al.*, in review), we did not make use of this possibility. The paternity exclusion probibility (PEP) was calculated from the CERVUS output for both datasets as one minus the parent-pair non-exclusion probability.

Table 1. Genotyping error estimates and paternity exclusion probability (PEP) for six *Campanula thyrsoides* loci, based on CERVUS 3.0.3 (Kalinowksi *et al.* 2007) computations using 22 mothers and 338 offspring without (RAW) and with (BIN) binning

	RAW			*BIN*		
	$N_{\mathrm{mismatch}}/N_{\mathrm{comparisons}}$	*Error*	*PEP*	$N_{\mathrm{mismatch}}/N_{\mathrm{comparisons}}$	*Error*	*PEP*
Campthy 1	9 / 313	0.0441	0.693	1 / 314	0.0058	0.632
Campthy 3	12 / 224	0.0778	0.737	3 / 231	0.0293	0.539
Campthy 5	17 / 144	0.1526	0.757	2 / 189	0.0219	0.558
Campthy 6	7 / 283	0.0385	0.683	0 / 290	0.0000	0.552
Campthy 9	6 / 323	0.0828	0.444	0 / 324	0.0000	0.357
Campthy 15	36 / 148	0.2802	0.791	2 / 148	0.0314	0.537
Overall		0.1127	0.999		0.0147	0.990

N_{mismatch} is the number of mismatching samples and $N_{\mathrm{comparisons}}$ is the number of compared samples. Error is the calculated error rate.

Each offspring was assigned to one of four different classes based on the threshold values (*T*) applied to their LOD-score and their Δ-score (Bacles & Ennos 2008), where Δ is the difference between the highest and second highest LOD-score. (i) LOD ≤ 0: immigrated; (ii) 0 < LOD < *T*: unassigned, potentially immigrated; (iii) LOD > *T* and Δ < *T*: unassigned, multiple fathers possible; (iv) Δ > *T*: assigned to a specific father.

Compared to the RAW dataset, the BIN dataset showed 88 more assigned offspring. Both the number of offspring assigned to fathers and the number of offspring assigned as immigrants were higher in the BIN dataset. Based on this improvement, and since the BIN dataset was assumed to be more reliable than the RAW dataset, only results of the BIN dataset are discussed henceforth.

Within-population pollen movement

We mapped the pollinations within the population as assigned by the paternity analysis and calculated the pollination distances, from which a pollination distance histogram was drawn. It is important to realise that the observed pollination distribution is partly dependent on gene flow processes and partly on the spatial distribution of individuals (Oddou-Muratorio *et al.* 2005; Van Rossum *et al.* 2011). We therefore tested whether the observed pollinations came from the same distribution as expected by random mating. As random mating distribution we used a variant of the distribution of distances of all realised pollinations as in the paternity analysis assignments with each father virtually pollinating all other individuals for each realised pollination. We used Kolmogorov-Smirnov tests to compare the distribution (i.e. location and shape) of distances grouped into distance classes. We also used Mann-Whitney *U*-tests to see whether the means (i.e. location) differed between distributions (Sokal & Rohlf 1995). Furthermore, we constructed windrose diagrams indicating the direction of pollinations and checked whether these directions deviated from random mating. R statistical package (R Development Core Team 2009) was used for these non-parametric statistical tests and the windrose was constructed using the package *circular* v0.3-8 (Lund & Agostinelli 2007).

Reproductive success

To test more specifically whether the isolation of mother plants affected the effective pollen sources, we regressed the average distance of all pollinations to a specific mother plant with the distance to the nearest neighbour of that mother plant or with the average distance to source individuals as explanatory factor to test whether isolation of mother plants could explain pollination distance. We also investigated with Kolmogorov-Smirnov tests whether the distribution of pollination distances differed from the distribution of nearest neighbour and average distances. Mann-Whitney *U*-tests were used to investigate whether the pollination distances were significantly larger than the distances to the nearest neighbour or whether they were significantly different from the average distance.

To test whether the isolation of mother plants affected the diversity of pollen sources, we calculated a source diversity index, calculated as one minus the number of different fathers contributing to a mother's offspring divided by the number of assigned offspring for that mother. This source diversity index was used in generalised linear models with a binomial error distribution (*glm* function in R; R Development Core Team 2009) applying the distance to the nearest neighbour and the average distance to source individuals as independent variables. Since a substantial part of the offspring per mother had only few assigned fathers, we conducted the analysis only with those mothers having > 5 assigned offspring.

To investigate male mating success, we calculated the relative reproductive success of each father plant as the proportion of pollinations by that father plant out of the total number of pollinations. For each mother plant, we also calculated the proportion of pollinations by a particular father. We then averaged these proportions of pollinations by a particular father over all mother plants. Both measures of male mating success were again fitted using a generalised linear model with a binomial error distribution and with distance to nearest neighbour and average distance to mother plants as independent variables in order to test whether inter-plant distances could explain male mating success.

Immigrant pollen flow

Estimation based on dispersal curve

We fitted pollination frequencies per distance class to inverse power, Weibull, exponential and exponential power models (Streiff *et al.* 1999; Pluess *et al.* 2009) using the *nls* and *eval* functions in R (R Development Core Team 2009). The inverse power model fitted best. For pollination frequencies, we used distance classes of 2 m, which resulted in a balanced resolution versus sample size. In the inverse power function

(1) $f(d) = \frac{a}{d^b}$,

f(d) is the frequency of occurrences in distance class *d*, while *d* is distance, and *a* and *b* are optimisation parameters.

The fitted model was used to estimate the fraction of migrant pollen flow. We integrated the area under the curve of the fitted model from a threshold range to the distance of the farthest population on the Schynige Platte, which was population no. 24 at 1,408 m distance (Fig. 1), to determine the proportion of theoretical long-distance dispersal. The threshold range was determined as (1) the farthest distance between two flowering individuals within the experimental population, i.e. 80 m, and (2) the shortest distance to a neighbouring population, i.e. 260 m.

Immigrant assignment

Rare or unique alleles (frequency < 0.01 in the mother samples) can be used to assign offspring to specific source populations. We assigned offspring which CERVUS indicated as immigrants to foreign pollen sources using genetic data from 5 microsatellites (Campthy 1, Campthy 3, Campthy 5, Campthy 6, Campthy 9) for 12 individuals of each of the 24 populations, sampled from flowering plants in the summer of 2006 (Frei *et al.*, in review). For each offspring assigned as immigrant, we calculated a probability ranking value of source populations using the following formula:

(2) $\sqrt{a(1 - D_{prop})}$,

where *a* is the frequency of the rare or unique allele in one of the 24 populations and D_{prop} is the inverse of the proportional distance from a source population to the study population (0–1; scaled to the furthest possible distance on the Schynige Platte).

Additionally, we performed an independent method to assign immigrants to their source population with the program GENECLASS2 (version 2.0.h, Piry *et al.* 2004). Based on the allele frequencies in the 24 populations on the Schynige Platte mountain plateau, this program ranks populations according to the probability that it produced the offspring that were classified by CERVUS as immigrants. We used the option to assign/exclude populations as origin of single individuals with an assignment threshold score of 0.05, using the Bayesian method according to Rannala and Mountain (1997) and the probability computation according to Paetkau *et al.* (2004) with 10,000 Monte-Carlo resamplings and a Type I error of 0.01. Whereas the reference population data consisted of diploid genotypes, we removed the mother alleles from the immigrant genotypes. Because GENECLASS2 does not take into account the distance to the source population, we multiplied the probability ranking values that an offspring originated from a particular population with the inverse proportional distance, D_{prop}, from that population to the study population.

Fluorescent pollen dispersal experiments

Pollen dispersal distances were measured in the study population on 11 July 2008 and 13 July 2009 using fluorescent pollen analogues (Radiant Colour, Houthalen, Belgium). In both years, the day of observation was overcast with mild temperatures (ca 15°C) and no wind, and sparse raindrops in the late afternoon. There was abundant insect activity. All individuals with inflorescences were flowering on the measuring days, with the majority of flowers being receptive. In both years, the position of each flowering individual was mapped. Three individuals from different parts of the population were selected as donors, and fluorescent pollen analogues of different colours (red, yellow, blue) were applied to the stamina of each open flower directly after dawn. Pollinators, mainly bumblebees, transferred the pollen analogues to other flowering individuals during the day. After sunset, fluorescent pollen could be traced on the flowers of *C. thyrsoides* using UV torches and pollination events were recorded.

In order to test for differences in distribution of the paternity analysis and the fluorescent pollen experiments, we made pairwise comparisons between both realised and expected pollination distributions of the paternity analysis and the two fluorescent pollen dispersal data sets using Kolmogorov-Smirnov and Mann-Whitney *U*–tests, adjusting for multiple testing with Bonferroni correction.

Results

Paternity analysis

No identical multilocus genotypes were found among the 22 flowering individuals sampled in the study population on the Schynige Platte. Results of CERVUS reconfirm that all loci were in Hardy-Weinberg equilibrium. Among the 338 genotyped offspring, seven offspring had less than three loci scored and were excluded from the analysis. The BIN dataset (before solving remaining mother-offspring mismatches) mismatched for eight samples at one or more loci with their mothers. This amounted to an error rate of 0.0147, with complete absence of error for locus 6 and 9 (Table 1). Paternity exclusion probabilities amounted to 0.990 (Table 1). Six out of eight remaining inconsistencies could be overcome by deleting one of the homozygous alleles in the offspring. The remaining two pairs were not solvable and gel photos showed that genotyping was clear and correct. Therefore, mutations could have caused these inconsistencies and we left these two inconsistencies in the dataset. CERVUS assigned 114 offspring (34.4%) to specific father plants from the population and 25 offspring (7.6%) as immigrants (Table 2).

Table 2. Paternal assignment of 331 offspring sampled from 22 mother plants of *Campanula thyrsoides* to four classes (Immigrated; Unassigned, potential gene flow into population; Unassigned, multiple fathers possible; Assigned to father) based on BIN data of six microsatellite loci in population 19 on the Schynige Platte using CERVUS 3.0.3 (Kalinowksi *et al.* 2007)

Assigment class	Definition	*N* (% of total)
Immigrated	LOD ≤ 0	25 (7.6%)
Unassigned, potential gene flow into population	0 < LOD < Thr	7 (2.1%)
Unassigned, multiple fathers possible	LOD > Thr & Δ < Thr	185 (55.9%)
Assigned to father	LOD > Thr & Δ > Thr	114 (34.4%)

LOD, Log of the odds ratio for a certain sample; Δ, Difference between the two highest LOD-scores; Thr, Threshold value determined by a simulation of offspring based on the same mother plants.

Within-population pollen movement

The mean pollination distance based on the paternity analysis was 16.2 m (Fig. 2; Table 3), the average distance of pollinations expected based on random mating was 27.9 m.

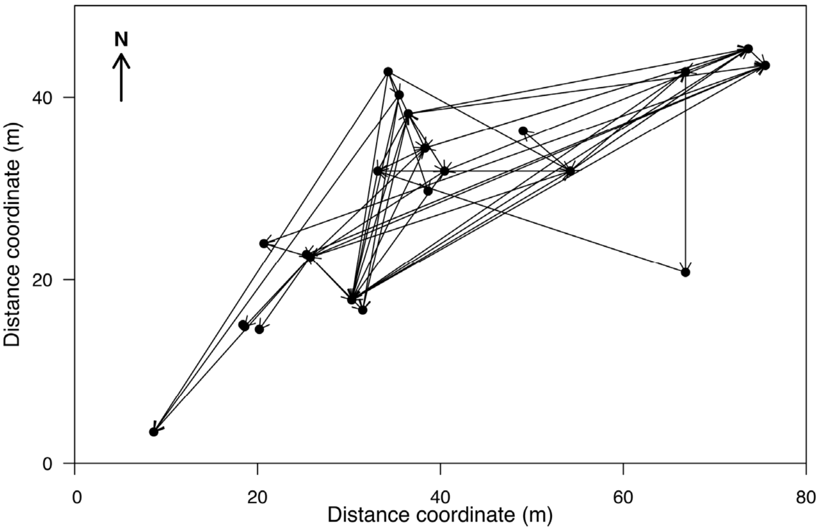


Fig. 2. Map showing the 22 flowering individuals of *Campanula thyrsoides* in the study population no. 19 on the Schynige Platte in 2007 with arrows indicating pollinations from father to mother as assigned by CERVUS (Kalinowksi *et al.* 2007).

Table 3. Average pollination distances and comparisons between realised and random pollination distances as well as pollination directions from three different years and two methods in *Campanula thyrsoides* (2007, paternity analysis; 2008 and 2009, fluorescent pollen experiments)

	n	dst_{pol} ± SD (m)	$distr_{pol} \sim distr_{random}$ Kolmogorov-Smirnov	$distr_{pol} \sim distr_{random}$ Mann-Whitney *U*	$dir_{pol} \sim dir_{random}$ Kolmogorov-Smirnov
Paternity analysis	114	16.2 ± 16.9	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Fluorescent pollen 2008	109	62.1 ± 22.8	$P < 0.001$	$P = 0.17$	$P < 0.0001$
Fluorescent pollen 2009	681	34.0 ± 24.7	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

N, sample size of detected pollinations; dst, average distance; distr, pollination distribution; dir, pollination direction; $_{pol}$, realised pollination; $_{random}$, random pollination; Kolmogorov-Smirnov test for differences in shape and location and Mann-Whitney *U*-test for differences in location.

Therefore, the distribution of realised pollinations showed significantly shorter distances than expected based on random mating (Mann-Whitney *U*-test, Fig. 3; Table 3).

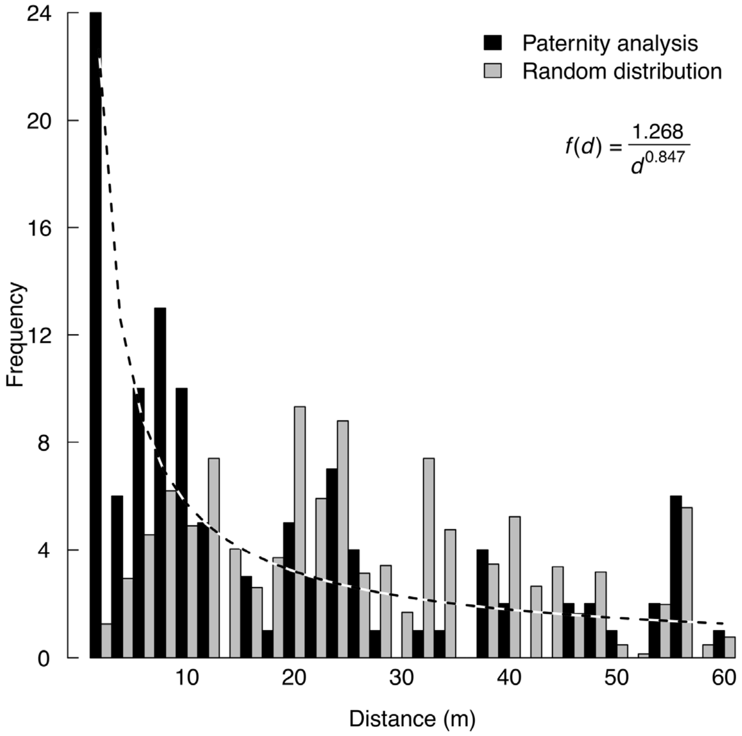


Fig. 3. Histogram of pollination frequencies in *Campanula thyrsoides* from the paternity analysis (black bars) and from a distribution based on random mating (grey bars) based on distance classes of 2 m. The dotted line indicates the model fit through the paternity analysis data.

The average distance of mothers to assigned fathers could be explained by both distance to nearest neighbour ($N = 20$; $F = 9.11$; $P = 0.0074$; $R^2 = 0.30$) and average distance to other plants ($N = 20$; $F = 16.04$; $P = 0.0008$; $R^2 = 0.44$). Kolmogorov-Smirnov and Mann-Whitney *U*-tests indicated that shape and location of both compared distributions differed ($P < 0.001$ for all four tests; data not shown), with the pollination distances being larger than distances to nearest neighbour and generally shorter than average distances to other plants. Pollinations occurred in all directions except northwards (Fig. 4). These directions showed a distribution being different from random mating (Table 3), which predicted pollinations mainly in northeast and southwest directions due to more plants positioned along this axis. Two pollinations were due to self-fertilisation.

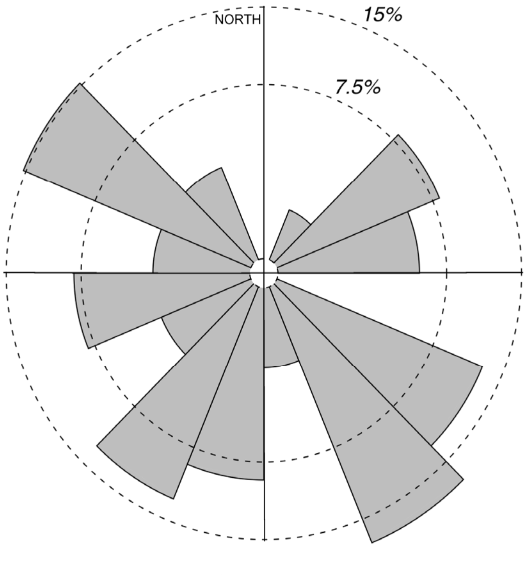


Fig. 4. Windrose with size of grey areas indicating percentages of pollinations in *Campanula thyrsoides* in specific directions within the study population no. 19 on the Schynige Platte in 2007 as assigned by CERVUS (Kalinowksi *et al.* 2007).

Reproductive success

For mother plants, the logistic regressions of source diversity index were not significant for distance to the nearest neighbour ($n = 6$; Chi^2 = 2.1; $P = 0.15$) and for the average distance to source individuals ($n = 6$; Chi^2 = 3.05; $P = 0.08$). This could well be due to sample size, as only six mothers had > 5 assigned offspring. Relationships tended to be negative, suggesting that the diversity of fathers diminishes with increasing isolation of mothers.

A total of 14 out of 22 (64%) potential pollen donors in the study population were found to contribute to pollen flow, of which two sired one offspring and the remaining 12 sired 2–33 offspring. Overall proportional male mating success ranged from 0.00–0.29, indicating that the most successful father sired 29% of the offspring assigned by paternity analysis. Male mating success calculated as the average of proportional pollinations of mother plants ranged from 0.00–0.50. These two measures of male mating success could neither be explained by distance to nearest neighbour ($P = 0.69$ and $P = 0.90$, respectively) nor by the average distance to other plants ($P = 0.80$ and $P = 0.73$, respectively), indicating that variability in male mating success was not due to distance effects.

Immigrant pollen flow

Estimation based on dispersal curve

The inverse power model had the highest log-likelihood (-71.0) compared to other models. Both optimisation parameters $a = 1.268$ and $b = 0.847$ were statistically significant ($P < 0.001$). The fraction of extrapolated area under the curve which defines the immigrant pollen ranged from 23–36% of total pollinations, which is much higher than the paternity analysis estimate of 7.6 %.

Immigrant assignment

We subtracted the mother genotype from the 25 offspring of which the father was assigned as originating from outside the study population. This resulted in 14 individuals containing one or more alleles which were either rare or absent in the study populations. With the probability ranking function that integrated allele frequencies in the 24 populations with the distance to these populations, each of these fourteen offspring could be assigned to a surrounding population with variable probability ranking values (Table 4) and all five first-rank source populations were neighbouring the study population (Fig. 1).

Table 4. Assignment of 25 samples classified by CERVUS (Kalinowksi *et al.* 2007) as immigrants to *Campanula thyrsoides* populations on the Schynige Platte by (i) the rare-allele method and (ii) by GENECLASS2 (Piry *et al.* 2004), both adjusted with the relative distance to the study population (see Materials and methods)

	Rare alleles × relative distance					GeneClass2 × relative distance			
SampleID	Locus-Allele	Pop 1	A_{pop1}	Pop 2	A_{pop2}	Pop 1	A_{pop1}	Pop 2	A_{pop2}
62	9-171	12	0.39	15	0.36	10	0.50	14	0.34
92	-	-	-	-	-	21	0.83	10	0.78
107	-	-	-	-	-	20	0.47	13	0.36
195	-	-	-	-	-	17	0.12	12	0.08
212	9-182	**10**	0.30	18	0.16	**10**	0.32	19	0.17
218	9-182	**10**	0.30	18	0.16	**10**	0.31	19	0.17
219	-	-	-	-	-	21	0.83	10	0.78
225	9-165	17	0.61	12	0.42	10	0.77	21	0.77
226	9-165	**17**	0.61	12	0.42	10	0.45	**17**	0.44
251	5-125	20	0.59	18	0.40	10	0.52	13	0.32
251	9-182	**10**	0.30	18	0.16	-	-	-	-
262	9-182	**10**	0.30	18	0.16	13	0.16	**10**	0.16
277	-	-	-	-	-	-	-	-	-
283	9-182	**10**	0.30	18	0.16	**10**	0.32	19	0.17
284	6-159	15	0.54	**12**	0.42	**12**	0.88	21	0.83
285	9-182	**10**	0.30	18	0.16	**10**	0.31	19	0.17
286	9-182	**10**	0.30	18	0.16	**10**	0.31	19	0.17
288	9-182	**10**	0.30	18	0.16	**10**	0.31	19	0.17
289	9-182	**10**	0.30	18	0.16	**10**	0.31	19	0.17
302	-	-	-	-	-	10	0.78	21	0.76
322	-	-	-	-	-	10	0.76	13	0.56
324	-	-	-	-	-	10	0.35	7	0.25
326	-	-	-	-	-	10	0.49	19	0.39
336	-	-	-	-	-	17	0.20	12	0.17
340	-	-	-	-	-	17	0.21	12	0.17
344	-	-	-	-	-	10	0.49	19	0.4

The first and second-best populations are given for both methods, including their probability ranking value. Pop, Population; *A*, Probability ranking value. Identical source populations for both methods are indicated in bold.

We also assessed which population could be the pollen source of immigrants by using an assignment computation in GENECLASS2 combined with a distance factor. This resulted in a separate classification of six first-rank populations. The rare/unique allele assignment shared first or second rank with the GENECLASS2 method for 11 out of 14 offspring (Table 4).

Fluorescent pollen dispersal experiments

The years 2008 and 2009, during which the fluorescent pollen experiments were performed, counted 93 and 83 flowering plants, respectively. Pollinators carried fluorescent pollen from the three selected donor plants to 30 and 57 mother plants with 109 and 681 pollinated flowers, respectively (Appendix 2). The realised average distances were 62.1 m for 2008 and 34.0 m for 2009 (Table 3). Random mating pollination distances were on average 60.7 m for 2008 and 48.9 m for 2009. The distribution of realised pollinations differed in shape from random mating, but their location did only differ in 2009. The distributions of the pollination directions differed from directions based on random mating in both years (Table 3).

When comparing pairs of distributions from the paternity analysis and the two fluorescent pollen experiments, all three pairs of realised distributions as well as distributions expected with random mating differed from each other in shape as well as in their mean (i.e. location) with $P < 0.0001$ after adjusting for multiple tests (data not shown).

Discussion

To summarise the answers to our four questions, we found (i) a considerable fraction of offspring fertilised with foreign pollen which (ii) originated from neighbouring populations. (iii) The spatial location affected the pollination distances to mothers but did not influence male mating success. (iv) The paternity analysis showed shorter effective pollination distances than the fluorescent pollen analogues.

Immigrant pollen flow

The paternity analysis successfully assigned a considerable amount of the investigated *Campanula thyrsoides* offspring (7.6%) as immigrant (Table 2), indicating that effective gene flow into the population is substantial. This value is lower than the estimated 23–36% based on the area under the curve of the inverse power model fitted on the paternity analysis data. Geng *et al.* (2008) also found that their model estimated a substantially higher pollen flow compared to results of the paternity analysis in a study on the mangrove species *Kandelia candel*. There can be alternative reasons for this discrepancy. One explanation could be that, since several samples were neither assigned to fathers nor assigned as immigrants, the number of detected immigrants is actually a minimum value and could be as high as 9.7% if the unassigned samples with a LOD-score below the threshold value would be added (Table 2). However, this proportion is still much below the model estimate. The paternal exclusion probability was 0.990, and with 22 mother plants this amounts to $0.990^{22} \approx 0.80$ as fraction of true assignments (Bacles & Ennos 2008). Therefore, the amount of cryptic gene flow could be up to 20%, amounting to a maximum immigration rate of 29.7%, which is in the range of the model estimate.

As an alternative, perhaps more likely, explanation, the model is fitted based on within-population pollinations, and the assessment of immigration based on the model is therefore an extrapolation based on the assumption that the within-population characteristics continue outside the population. This is clearly wrong as the density of plants per definition declines sharply at the boundary of the population due to unoccupied area. Therefore, we are more confident in the results of the paternity analysis, which present a minimum of pollen flow into the population, and we consider the model estimates as overestimations.

The spatial isolation of *C. thyrsoides* populations on the Schynige Platte is less extreme as elsewhere in its distribution, which probably increased the proportion of immigrants in our study compared to the average population throughout its range. The use of the atypical field situation in this study can be seen as a drawback to understand pollen flow in *C. thyrsoides*, but its advantage was that we were able to assess the influence of source population distances on pollen immigration. The assignment of source populations by means of the rare allele method as well as with the program GENECLASS2 indicated that source populations included the immediately surrounding populations and that inter-population pollination distances can be up to 954 m (Population no. 21, Fig. 1). Bumblebee flight activity has been reported to be within a range of ~650 m (Osborne *et al.* 1999; Darvill *et al.* 2004). Thus, our largest observed pollination distance extends further but is still reasonable.

Within a radius of < 1 km the potential source pollinations would include a total of 13 populations on the Schynige Platte (no. 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22; Fig. 1) of which six have been found to contribute pollen in this study (Table 4).

The fraction of immigrant pollen flow ranges widely among animal-pollinated species and populations (Ashley 2010). Miyazaki and Isagi (2000) found the fathers of all investigated offspring (a total of 124) from four mother plants of *Heloniopsis orientalis* to be from inside the population (ca 30 × 30 m), where surrounding populations were located at >200 m. Kameyama *et al.* (2001) found that gene flow among subpopulations of *Rhododendron metternichii* var. *hondoense*, which were separated by approximately 50 m, was low (0–2%). The monocarpic perennial *Centaurea corymbosa* also showed little pollen flow among six populations within 3 km^2 (Hardy *et al.* 2004). Substantial pollen flow among widely spaced individuals has also been documented, within stands as well as among populations. In the animal-pollinated Cactaceae *Polaskia chichipe* 27% of pollinations was between populations, with three pollinations exceeding 1,000 m (Otero-Arnaiz *et al.* 2005). A single population of the shrub *Prunus mahaleb* showed 9.5% of the pollen flow exceeding 1,500 m (García *et al.* 2005). Kamm *et al.* (2009) even found 10% of pollen donors in *Sorbus domestica* exceeding 2,000 m distance to the mother plant. In the context of findings from the mentioned and other (Ashley 2010) studies, among-population pollen flow in *Campanula thyrsoides* falls within the range exhibited by other species. We conclude that the populations on the Schynige Platte, occupying an area of ca 4.4 km^2, are well-connected by pollen dispersal. However, other natural populations of *C. thyrsoides*, which are generally more isolated, may experience less immigration of pollen and therefore probably stronger differentiation, as geographic isolation is generally more extreme.

Within-population pollen movement

Unequal contributions to reproduction may affect the effective population size and therefore may increase the rate of fixation and loss of alleles (Oddou-Muratorio *et al.* 2005). The location of individuals in a population could translate into variability in reproductive success, with isolated mother plants receiving pollen from donors further away and isolated fathers having fewer pollen donations (Oddou-Muratorio e*t al.* 2005). As a first indication of spatial effects on pollination, the distribution of realised pollinations showed shorter distances than expected based on random mating (Fig. 3; Table 3). Furthermore, variability in the average distance of mothers to assigned fathers could be explained by both nearest neighbour and average distance. This indicates that distance from mother to father plants is a limiting factor for pollinations, which can be explained (i) by pollinators depositing most pollen grains on the first few individuals visited after the source plant and (ii) by passive loss of pollen during flight (Van Rossum *et al.* 2011 and references therein). The distribution of pollination directions also differed in shape from the distribution based on random mating (Table 3), which seems to be due to longer distances between plants on the southwest-northeast axis, and therefore fewer pollinations, than in northwest-southeast directions. Thus, spatial positioning of mother plants clearly affected pollination distances.

The paternity analysis attributed only two offspring from different mother plants to self-fertilisation, which is in line with breeding experiments which showed that *C. thyrsoides* has almost complete self-incompatibility (Ægisdóttir *et al.* 2007a), although apparent self-fertilisation could also be explained by cryptic gene flow.

Reproductive success

Although the pollination of mother plants was affected by their degree of isolation from other plants, the degree of isolation of potential fathers showed ambiguous results. More than half of the 22 fathers were found to contribute to pollinations among the 331 analysed samples. Five out of eight individuals that did not contribute pollen had a position on the periphery of the population (results not shown), which suggests some effect of distance on male mating success. There also was great inequality in the number of offspring sired by different fathers, and a single father (individual 14; Fig. 1) even pollinated 33 offspring with 11 different mothers. The second-most successful father (individual 17) pollinated 17 offspring with eight mothers. Both fathers had central geographical positions in the population. This suggests strong effects of degree of isolation, but in contrast to expectations, pollination success of fathers as the proportion of all pollinations was not dependent on distance to nearest plants or on the average distance to plants. Neither was the pollination success as the average of proportions of offspring sired by a specific father per mother plant dependent on distance to nearest plant or on average distance to plants.

Although studies generally show strong effects of distance on male mating success (e.g. Burczyk & Prat 1997; Oddou-Muratorio *et al.* 2005), a similar situation to our results was found for *Chamaelirium luteum* (Smouse *et al.* 1999) where pollinations decreased with increasing distance from a focal mother plant to a potential father, but where male mating success could not be explained by distance or reproductive morphology. Since the obvious variability in male mating success in this study did not depend on the degree of isolation in the population, we conclude that this result must be due to other factors, for instance unknown topographic effects or number of flowers produced. Another reason for the absence of relationships could be that, for both measures of pollination success, the variable number of offspring analysed per mother could have affected the analysis, as four of the 20 mothers had < 10 offspring analysed. Variability in flowering phenology, which leads to deviations from the optimal pollen presentation time in flowering individuals (Burczyk & Prat 1997; Kitamoto *et al.* 2006), in combination with variable weather conditions affecting pollinator activity (Lundberg 1980), may also have played a role.

Fluorescent pollen versus paternity analysis

The estimates of pollen movement within and into the study population differ quantitatively between the fluorescent pollen experiments and the paternity analysis, with the former showing far larger average pollination distances (Table 3). This could be due to the different methods as well as to variable number and positioning of flowering plants. An important distinction between the paternity analysis and the fluorescent pollen experiments is that the former assesses the outcome of dispersal and effective pollination (i.e. pollen flow), whereas the latter is confined to pollen dispersal alone (Van Rossum *et al.* 2011). It could be speculated that fluorescent pollen dispersal measurements are likely to overestimate gene flow at short distances, since effects such as self-incompatibility and inbreeding depression are undetectable by this method (Van Rossum *et al.* 2011). However, although self-incompatibility is strong, sister-mating yields normal amounts of seeds and normal offspring development (Ægisdóttir *et al.* 2007a). Moreover, pollen dispersal distances were found to be larger, not smaller, in the two fluorescent pollen experiments than in the paternity analysis. Comparable to our study, fluorescent pollen was found to reach larger distances than normal pollen in the bumblebee-fertilised *Erythronium grandiflorum* (Thomson *et al.* 1986). Our results indicate that estimates of reproductive success from various methods should be judged with care.

Concerning year-to-year variability in number and positioning of flowering plants, inter-plant distances were smaller in the years 2008 and 2009 due to a higher density of plants, but the overall area occupied by the population of flowering plants was much larger in these years. Therefore, although higher densities of flowering individuals reduce pollinator foraging distance (Fenster 1991; Schnabel & Hamrick 1995; Kameyama 2001), the larger area would increase pollination distance compared to the year 2007. According to our results, this area effect may be stronger than the density effect. Relating this to bumblebee flight behaviour, it may still hold true that pollinators generally fly from plant to neighbouring plant, but the (fluorescent) pollen load of a single pollinator may be so high (especially in foraging species such as bumblebees) as to mark a long series of receptor plants, carrying (fluorescent) pollen over long distances from the donor plant (Darvill *et al.* 2004; Van Rossum *et al.* 2011). Additionally, the observed inter-annual variability may also be due to different amounts of applied fluorescent pollen, since with more applied fluorescent pollen, the pollination distances between plants should increase as more pollen is foraged by pollinators.

The paternity analysis captured pollinations across the whole flowering season, whereas measurements of fluorescent pollen dispersal were conducted over a single day. The latter method may therefore have missed to capture rare dispersal events (Slatkin 1987; Bullock *et al.* 2006), but against this expectation, our results showed much larger pollination distances for fluorescent pollen than for the paternity analysis. Fluorescent pollen dispersal measurements may also show a strong bias in dispersal events which may be related to variability in floral phenology (Burczyk & Prat 1997; Kitamoto *et al.* 2006), or weather conditions affecting pollinator abundance and activity (Lundberg 1980). Even between the two fluorescent pollen experiments, the number of pollinations was more than sixfold in the year 2009 compared to the year before, which may be due to differences in pollinator abundance or weather-related activity. The position of the father plants chosen for the fluorescent pollen application may also have played a role, although we chose similar positions in both years. To conclude, the application of paternity analysis versus fluorescent pollen analogues may yield strongly diverging measurements, but it is also likely that temporal variability in environmental and distribution-related conditions affects the results.

Conclusion: the role of monocarpic perenniality

The results of the paternity analysis clearly indicate considerable gene flow into the population by means of long-distance pollen dispersal from other source populations. This suggests that connectivity among populations on the Schynige Platte is high. The among-population differentiation on the Schynige Platte is substantial with $G'_{ST} = 0.32$ (Frei *et al.*, in review), which seems to contradict this high rate of gene flow. However, as mentioned earlier, the specific life history of *C. thyrsoides* can account for this seeming contradiction. Populations of annual species are usually highly differentiated due to selfing and temporal limitations to mating (Loveless & Hamrick 1984; Vitali*s et al.* 2004). Likewise, the monocarpic perennial life cycle of *C. thyrsoides* limits mating possibilities, since less than 10% of plants in a population flower in a given year. Although genetic diversity can be kept high through efficient pollen flow among populations and subsequent storage of genes in dormant rosettes, the limited mating possibilities cause a reduced effective population size and subsequent among-population differentiation (Loveless & Hamrick 1984; Vitalis *et al.* 2004). The outcrossing behaviour of *C. thyrsoides* is important in retaining genetic diversity (Ægisdóttir *et al.* 2009), but the size- and microsite-dependent flowering and variance in growth rates likely causes considerable generational overlap, with the effect that the probability of sib-mating is strongly reduced (Kuss *et al.* 2008b). This desynchronized flowering of cohorts (Kuss *et al.* 2008b) further leads to the situation that in each year a partly random subset of rosettes will flower, with genetic diversity of these flowering individuals being high as they originate from a range of years. The flowering individuals may thus present high genetic diversity, and the pollen flow may likewise be high among populations. However, the flowering individuals present only a very restricted part of the overall genetic diversity stored in a population.

Migration is a strong force in reducing the level of differentiation, and only few migrants per generation are theoretically needed to prevent among-population differentiation (Conner & Hartl 2004). However, the theoretical study by Vitalis *et al.* (2004) as well as the results of our current experimental study suggest that in monocarpic species with long generation times even multiple migrants per generation cannot prevent differentiation among populations. If the estimated 7.6% immigration would have been constant over the years 2005–2009, the number of flowering individuals with foreign genes would have ranged between 1.7–8.0. Therefore, populations of monocarpic species can be considerably differentiated even though gene flow among populations can be substantial. This may be the first experimental study showing the importance of monocarpic perenniality for population genetic diversity and differentiation.

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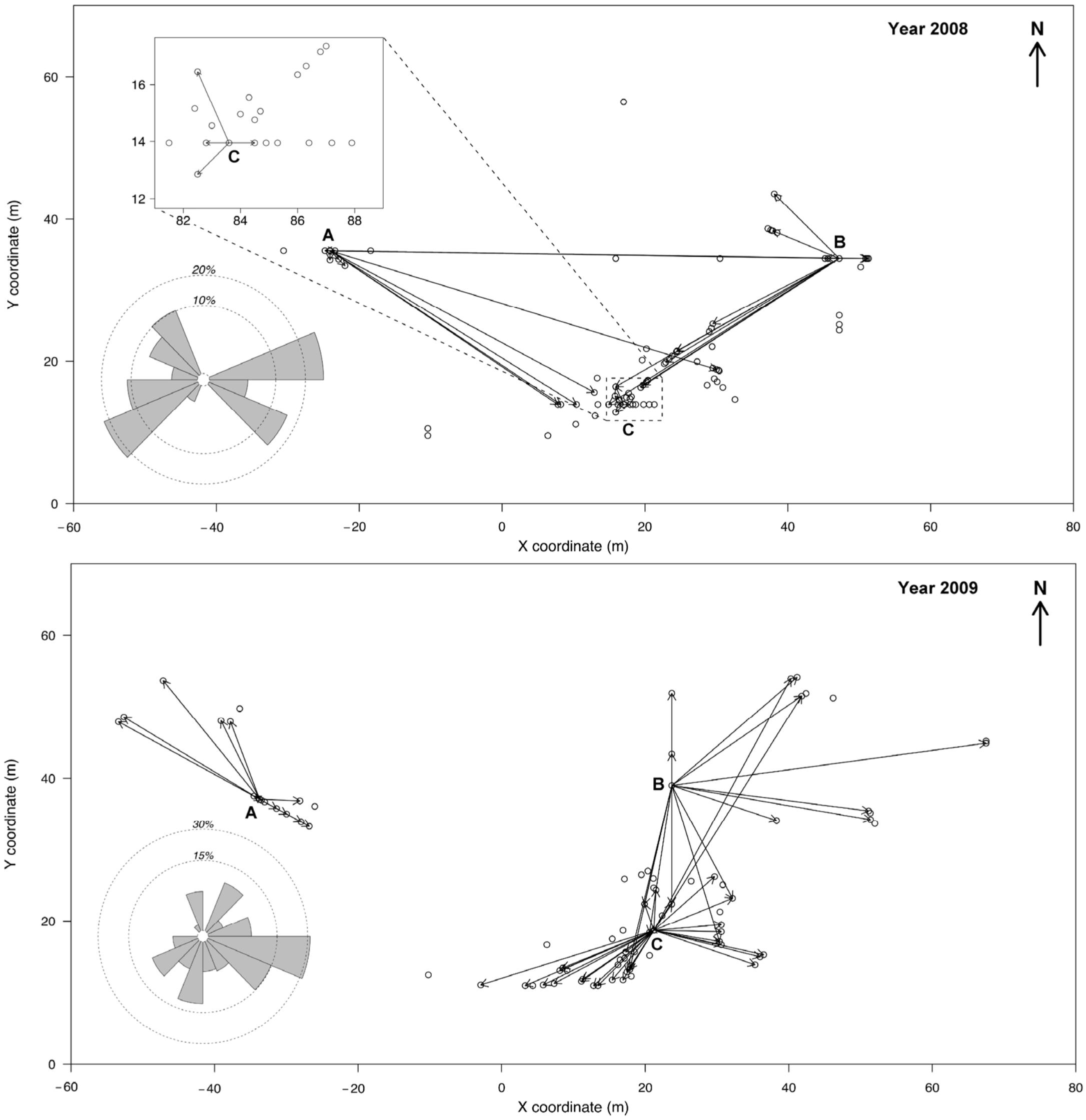
Appendix

Appendix 1. Binning applied to alleles of different *Campanula thyrsoides* loci (Ægisdóttir *et al.* 2007b)

Locus	Alleles binned
Campthy1	160; 162
Campthy3	127; 129; 131
Campthy3	147; 150; 153; 155
Campthy5	111; 113; 115
Campthy5	117; 120; 123
Campthy6	151; 153
Campthy6	161; 163; 165
Campthy6	167; 170
Campthy9	161; 163
Campthy9	184; 186
Campthy15	172; 174
Campthy15	176; 178

Allele codes indicate their lengths in basepairs.

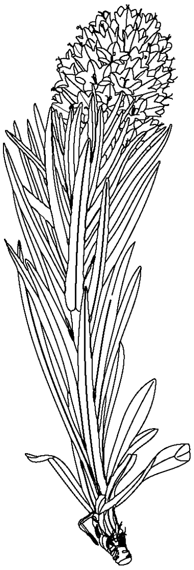


Appendix 2. Maps showing the pollinations as observed using fluorescent pollen applied to three flowering individuals (A, B, C) each in 2008 (top) and 2009 (bottom). The scale and coordinates of both maps is similar (also to Fig. 2). The inset in the map shows a windrose with size of grey areas indicating percentages of pollinations in specific directions.

Chapter 6

Dispersal and microsite limitation of a rare alpine plant

Eva S. Frei, J.F. Scheepens & Jürg Stöcklin



Summary

Knowledge on the limitation of plant species' distributions is important for preserving alpine biodiversity, particularly when the loss of alpine habitats due to global warming or land use changes is faster than colonisation of new habitats. We investigated the potential of the rare alpine plant *Campanula thyrsoides* L. to colonise grassland sites of different suitability on a small mountain plateau in the Swiss Alps. A total of 15 experimental sites were selected according to their differences in habitat suitability for adult *C. thyrsoides*, which was measured by the Beals index. At each site we applied a disturbance treatment, added seeds at different densities, and monitored the survival of seedlings over two consecutive years. The number of surviving seedlings was not positively related to habitat suitability for adult *C. thyrsoides*. Furthermore, *C. thyrsoides* appears to be strongly dispersal limited at the regional scale, because seed addition to unoccupied habitats resulted in successful germination and survival of seedlings. Since an increase of seed density in already occupied sites did not affect the number of seedlings, we suggest that *C. thyrsoides* is microsite limited at the local scale. Microsite limitation is supported by the result that seedling survival of the species was enhanced in vegetation gaps created by disturbance. We conclude that *C. thyrsoides* may become endangered in the future if environmental changes cause local extinction of populations. An appropriate management, such as a disturbance regime for enhancing recruitment in existing populations, may ensure the long-term survival of this rare alpine plant species.

Key-words: *Campanula thyrsoides*, dispersal limitation, disturbance, habitat suitability, microsite limitation, seed sowing experiment

Introduction

The occurrence of suitable habitats and the dispersal ability are the two main factors that influence a plant's distribution at the regional scale (Münzbergova & Herben 2005; Bullock *et al.* 2006). Habitat limitation is caused by environmental factors such as climate, soil conditions and vegetation succession. Dispersal limitation is the intrinsic limitation of the plant itself to disperse seeds, to recruit seedlings successfully and to establish a fully self-sustainable population in a newly colonised habitat. There is a well-known evolutionary trade-off between the number of seeds and the seed size: high seed numbers increase the chance of dispersal to a distant suitable habitat, while heavier seeds increase the establishment probability of seedlings (see review in Moles & Westoby 2004). At the local scale, distribution is theoretically limited by the availability of either microsites or seeds (Münzbergova & Herben 2005). However, in nature, local distribution seems to be influenced by a complex interaction of microsite availability, seed limitation and inter- or intraspecific competition between seedlings (Svenning *et al.* 2008; García-Camacho *et al.* 2010; Egawa & Tsuyuzaki 2011).

In the European Alps, the heterogeneous topography of the landscape may limit the dispersal ability of plants (Körner 2003; Bacles *et al.* 2006). Environmental conditions in some alpine habitats are stressful for germination and seedling establishment of plants due to the short time available without snow-cover (Bonde 1968). In alpine ecosystems, seedling establishment occurs either from seeds in the seed bank or seed rain (Chambers 1995) and has been observed to be strongly dependent on the dispersal mode and the successional stage of available sites (Stöcklin & Bäumler 1996; Niederfriniger-Schlag & Erschbamer 2000). Disturbances resulting from natural and human causes may positively affect seedling recruitment and variation in the timing of germination may reflect differences in seed longevity and dormancy among alpine plant species (Chambers *et al.* 1990).

Recently, land use changes and global warming have led to a loss of suitable habitats for many alpine plant species and to an up-ward extension in the altitudinal range of several species (Walther *et al.* 2002; Rudmann-Maurer *et al.* 2008). In a fast-changing environment (e.g. global warming), plant species with a weak colonisation potential and limited dispersal need assistence in dispersal of their seeds (Primack & Miao 1992). Therefore, from the perspective of preserving alpine biodiversity, investigating the limiting factors of a species' colonisation potential may allow predictions of future range changes in alpine plant communities and the formulation of species-specific management strategies for rare plants (Franzén & Eriksson 2003).

In the present study, we investigated the dispersal ability and the potential of the rare alpine plant *Campanula thyrsoides* L. to colonise habitats of different suitability on a mountain plateau (Schynige Platte) in the Swiss Alps. *C. thyrsoides* is well suited to study distribution limitations at the regional and local scales for several reasons. First, *C. thyrsoides* has a very narrow ecological niche, i.e. the differences in its habitat requirements for humidity, nutrients, temperature and light were very small in a wide comparison of *C. thyrsoides* habitats (Wüest 2008). Therefore, the species has to disperse over long distances to reach unoccupied suitable habitats. Second, while a single individual plant of this species produces numerous seeds, the dispersal propagules have no morphological structures to support dispersal. Furthermore, pre-dispersal seed predation can cause complete seed loss (Kuss *et al.* 2007). These two facts suggest that the distribution of this species is dispersal as well as occasionally seed limited. Third, after colonisation of a new site, at least two individuals must reproduce at the same time in order to successfully establish a new population, because of the strong self-incompatibility and monocarpic life-cycle of *C. thyrsoides* (Ægisdóttir *et al.* 2007; Kuss *et al.* 2007).

We used a relatively new method, which is based on species co-occurrence data from vegetation relevées and a suitability index (Beals index value; Münzbergova & Herben 2004), to select a total of 15 experimental sites of different suitability for a seed sowing experiment with *C. thyrsoides*. Seed sowing experiments are a straightforward method for investigating the factors influencing a plant's distribution (Turnbull *et al.* 2000), and allowed us to test if germination and survival of seedlings are dependent on the habitat suitability of sites for adult *C. thyrsoides*. By adding seeds to unoccupied sites, we additionally tested for dispersal limitation at the regional scale. By sowing seeds in different densities to already occupied sites and by using control plots without seed addition, we tested for seed limitation at the local scale. Finally, by modifying the conditions for germination with a disturbance treatment, we tested whether microsite availability is enhanced by disturbance and whether disturbance would be an appropriate regime for conservation management of the rare *C. thyrsoides*. The following hypotheses were addressed in our study: (1) The higher the habitat suitability (Beals index) for adult *C. thyrsoides*, the higher the germination and survival rate of seedlings is. (2) Addition of seeds to unoccupied habitats results in successful germination and survival of seedlings. (3) Augmentation of seeds in already occupied habitats increases the number of seedlings. (4) The number of seedlings is positively related to a disturbance treatment.

Materials and methods

Study species and region

Campanula thyrsoides occurs in the European Alps, the Jura Mountains and in the Dinaric Alps (Aeschimann *et al.* 2004). Typical habitats of the species are subalpine and alpine grasslands to screes on limestone or carbonate-bearing schists. The plants require a moderately disturbed regime, which may be created naturally (open soil in steep topography) or by human land use. It is assumed that disturbances positively affect seedling establishment by creating suitable microsites and reducing competition from other plants (Kuss *et al.* 2007). Thus, the species may be locally abundant in disturbed areas such as road shoulders, where populations may expand locally (Kuss *et al.* 2008), while it is rare at the regional scale and protected in the majority of the Alpine countries (Moser *et al.* 2002). *C. thyrsoides* is one of the few monocarpic perennials in the Alps. The plants produce a dense spike composed of 50–200 yellow and bell-shaped protandrous flowers (Scheepens *et al.* 2011). They die after the production of 15,000–50,000 tiny seeds with no morphological adaptations for dispersal. Seeds are trapped by the whithered bracts and are only dispersed when wind, rain or animals shake the seeds out of the capsules. Modelling seed dispersal by wind resulted in 99.9% of the seeds being dispersed within 10 m distance to the mother plant.

As study region, we used a topographically highly structured mountain plateau (Schynige Platte, centered at 46° 39’ 12” N; 7° 54’ 42” E) in the Swiss Alps. The mountain plateau covers an area of about 10 km^2 and includes an altitudinal range of 1,800–2,100 m a.s.l. On the Schynige Platte, *C. thyrsoides* occurs in 24 populations mostly in grassland or steep screes, which are spatially separated by small forest patches. The geographic distances between population pairs range from 0.5–3.0 km.

Estimation of habitat suitability using relevé data

The Beals index of sociological favorability (Beals 1984) estimates the occurrence probability of a species at a distinct habitat using species co-occurrence data from vegetation relevées. This index can also be used as a suitability index (Beals index value), which describes the suitability of habitats for a given plant species (Münzbergova & Herben 2004). The threshold value of suitability for unoccupied habitats corresponds to the lowest Beals index value of all relevées where the target species was found. Therefore, all habitats with a higher Beals index than this threshold are considered as suitable and the higher the Beals index (range 0–1), the more suitable a habitat for the species is.

In 2008, 87 vegetation relevées were recorded on randomly chosen grid points and within natural populations of *C. thyrsoides* on the Schynige Platte, with each of them including an area of 49 m^2 (Wüest 2008). Suitable unoccupied habitats were identified based on species co-occurrence data using the relevées with and without *C. thyrsoides* of Wüest (2008) and 138 relevées of Fischer and Wachter (1991). For each of the 225 relevées, the Beals index was calculated according to the formula described in Münzbergova and Herben (2004).

Seed addition and augmentation experiment

In order to investigate the potential of *C. thyrsoides* to colonise grassland habitats of different suitability on the Schynige Platte, we selected 15 sites for a seed sowing experiment in such a way that they covered the entire spectrum of the Beals index as inferred from vegetation relevées described above (Table 1).

Table 1. Location and characteristics of the 15 experimental sites in a seed sowing experiment with *Campanula thyrsoides* on the Schynige Platte in the Swiss Alps

Site	Beals index	Habitat quality	Lat (N)	Long (E)	Altitude (m a.s.l.)	Exp	Slope (%)	Soil pH	Ri	Rco (%)	Gco (%)	Fco (%)	Dco (%)
1	0.099	low	167358	636149	1947	E	30	5.0	48	2	47	5	0
2	0.116	low	167153	636155	1950	NNE	20	4.5	62	10	42	10	3
3	0.132	low	167254	636254	1941	WNW	30	5.0	64	10	20	5	5
4	0.169	low	167744	636942	1945	S	35	4.5	75	1	47	5	7
5	0.171	low	166958	636147	1984	E	25	5.0	75	10	30	10	5
6	0.186	low	167146	636751	1876	SE	60	5.0	82	3	35	8	7
7	0.200	medium	166946	636067	2029	ESE	50	5.0	66	2	45	2	3
8	0.223	medium	167556	636155	1972	SE	30	7.0	51	15	40	10	2
9	0.251	medium	167322	635953	2040	SE	75	6.0	66	30	30	5	5
10	0.276	high	167184	635687	1901	SW	65	7.0	64	20	25	5	1
11	0.293	high	167176	636503	1928	SSE	80	5.0	64	7	25	8	13
12	0.305	high	167125	635690	1886	WSW	60	7.5	61	25	20	5	10
13	0.305	high	167143	635881	2005	SW	60	7.0	44	20	9	3	13
14	0.306	high	167090	635923	1996	WSW	75	7.0	48	15	28	10	14
15	0.318	high	167125	636547	1908	SE	100	5.5	70	15	30	5	8

Sites were grouped to habitat quality: low = unoccupied and unsuitable sites; medium = unoccupied, but suitable sites; high = occupied and suitable sites for *C. thyrsoides*. Suitability of sites was measured by the Beals index from vegetation relevées, each including an area of 49 m^2. Sites are ordered from lowest to highest Beals index. Lat, latitude (Swiss Grid); Long, longitude (Swiss Grid); Exp, exposition (E = East, N = North, S = South, W = West); Ri, species richness (number of species); Rco, rock cover (%); Gco, grass cover (%); Fco, cover of Fabaceae (%); Dco, cover of dwarf shrubs (%).

We then classified the sites into three groups of habitat quality according to their occupancy and suitability for adult *C. thyrsoides*: Six sites were established in habitats where *C. thyrsoides* naturally occurred, i.e. sites of high habitat quality. Nine sites were established in habitats unoccupied by *C. thyrsoides*. From these unoccupied habitats, three sites were considered as habitats of medium quality because they would be suitable for the species, i.e. Beals index was larger than the threshold of suitability. The remaining six sites are referred as habitats of low quality (Beals index lower than this threshold).

In September 2008, mature seeds from 18 different natural populations of *C. thyrsoides* on the Schynige Platte were collected and mixed. On 20 September 2008, seeds of this mixture were sown in the 15 experimental sites (Fig. 1). Within each of the sites, eight experimental plots of 50 cm x 50 cm were established, and disturbance and seed density treatments were randomly assigned to them in the following way: Four plots were disturbed by clipping the grasses and herbs to ground level and by scarifying the upper soil layer with a three-pronged fork to simulate the activity of small mammals (e.g. Edwards & Crawley 1999), while four plots remained undisturbed. Seeds were added in three different densities (300, 3,000 and 30,000 seeds) to six of the plots, while in two plots no seeds were added. Seedlings were counted during three censuses: one each at the beginning and end of the growing season in 2009 and a third at the end of the growing season in 2010. We also measured the diameter of three surviving seedlings in each plot.

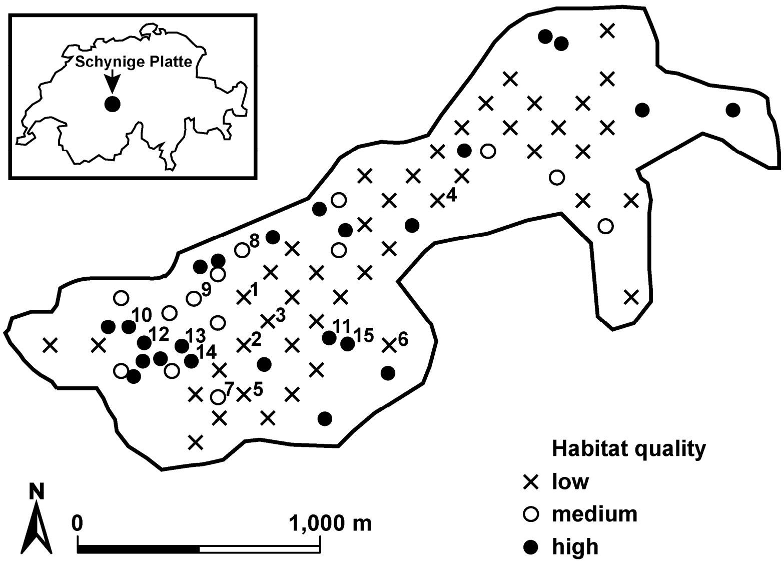


Fig. 1. Map of the study region on a mountain plateau (Schynige Platte) in the Swiss Alps with habitats of different quality for *Campanula thyrsoides*. Habitat quality: low = unoccupied and unsuitable sites; medium = unoccupied, but suitable sites; high = occupied and suitable sites for *C. thyrsoides*. Suitability of sites was measured by the Beals index (for details see Materials and methods). The 15 experimental sites used in the study are marked with numbers topleft of their locations. Other sites are vegetation relevées from 2008.

Mixed-effects model analyses

We analysed our data using linear-mixed modelling due to the hierarchical experimental design, with plots nested in sites. Linear-mixed modelling allowed us to handle binomial and Poisson data in the same framework as normal data, therefore transformation of the response variables was not necessary (Bolker *et al.* 2009). The germination rate (binomial error distribution) was fitted with a generalized linear mixed model (GLMM) and a *logit* link function (Crawley 2009). To assess the survival rate of seedlings, the proportion of surviving seedlings at the end of the first year to the number of germinated seedlings, and the proportion of surviving seedlings at the end of the second year to the number of germinated seedlings were calculated. Since the number of seedlings at the end of 2009 and 2010 showed a Poisson error distribution, these variables were also analysed with GLMMs, but with a *log* link function (Crawley 2009). In all GLMMs, we accounted for overdispersion by using a quasi-likelihood approach (Bolker *et al.* 2009). For the diameter of seedlings with a normal error distribution, we fitted linear mixed models (LMMs).

The simplest models included the *Habitat suitability* (Beals index), the two treatments *Disturbance* (2 levels) and *Seed density* (3 levels) as well as their interaction as fixed factors, while *Site* and *Plot* nested in *Site* were included as random effects. The sample size was $n = 90$ plots. In the more complex models ($n = 270$), a repeated measure analysis was used with *Time* (three censuses of measurements) included as an additional fixed factor to account for the time-dependency of seedling survival.

All models were fitted and tested using the statistical package R (R Development Core Team 2009). The model parameters were estimated with the *glmmPQL* function in the R-package MASS (Ripley 2005). Significance of the fixed effects was tested with *F*-tests (Faraway 2005), as recommended for GLMMs with overdispersion (Bolker *et al.* 2009). *A priori* contrasts were used to test for differences caused by different seed densities. As contrasts, low versus medium seed density and medium versus high seed density were tested. Likelihood ratio tests (Pinheiro & Bates 2000) were performed for testing random effects. Model assumptions (Pinheiro & Bates 2000) were checked using diagnostic plots constructed with the R-packages GGPLOT2 (Wickham 2010) and LATTICE (Sarkar 2009).

Additional analyses

To determine whether habitat suitability was related to environmental variables at the sites, such as slope or pH value of the soil, we performed a Pearson's correlation analysis between the Beals index and site characteristics (Table 1) using the statistical package R (R Development Core Team 2009). Soil pH was measured with a Hellige-Pehameter (AVM Analyseverfahren, Freiburg, Germany). To test for seed limitation in occupied sites, we compared the mean number of seedlings in control plots (without seed addition) to the one in experimental plots, by using one-sided *t*-tests.

Results

Effects of habitat suitability and time

The habitat suitability measured by the Beals index had a significant negative effect on germination rate, the number of surviving seedlings, but not on survival rates after one and two years (Tables 2 and 3).

Table 2. Summary of Generalized Linear Mixed Models (GLMMs) testing the effects of habitat suitability, disturbance treatment and seed addition density on germination, seedling diameter and survival of *Campanula thyrsoides* in a seed sowing experiment

Source of variation	Model effects		Source of variation	Model effects	
	df	Test statistics		df	Test statistics
Germination rate			Seedling diameter		
Habitat suitability	1	10.83**	Habitat suitability	1	0.90
Disturbance	1	12.53***	Disturbance	1	13.0**
Seed density	2	2.99(*)	Seed density	2	4.19*
Contrasts low vs. medium		-0.33	Contrasts low vs. medium		0.62
Contrasts medium vs. high		-2.43*	Contrasts medium vs. high		2.75**
Disturbance:Seed density	2	1.83	Disturbance:Seed density	2	0.46
Site	1	17.35****	Site	1	6.35**
Survival 2009			Survival 2010		
Habitat suitability	1	0.13	Habitat suitability	1	0.47
Disturbance	1	4.23*	Disturbance	1	6.49*
Seed density	2	1.1	Seed density	2	0.34
Contrasts low vs. medium		1.07	Contrasts low vs. medium		0.57
Contrasts medium vs. high		1.57	Contrasts medium vs. high		1.09
Disturbance:Seed density	2	1.87	Disturbance:Seed density	2	0.87
Site	1	0.82	Site	1	3.97*

df, degrees of freedom. Test statistics are F-values for fixed effects (*Habitat suitability, Disturbance*, *Seed density*, *Disturbance:Seed density*), t-values for *a priori* contrasts, and ChiSquare values for random effects (*Plot, Site*). Habitat suitability was measured by the Beals index (for details see Materials and methods). Plots ($n = 90$) are nested in sites. Asterisks represent significance levels: (*) $P < 0.08$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

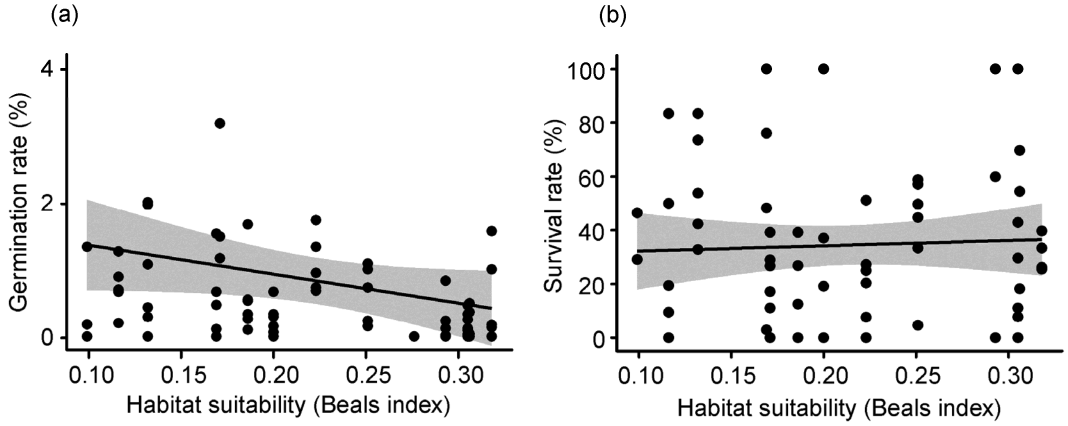


Fig. 2. Seedling establishment of *Campanula thyrsoides* in a seed sowing experiment with **a** germination rate at the beginning and **b** survival rate at the end of 2009 as a function of habitat suitability (measured by the Beals index; for details see Materials and methods). The lines represent the fit obtained by the model analyses and the grey shaded areas are the 95% confidence intervals.

Germination rate was lowest (mean 0.18%; Table 4) in occupied and suitable sites, and increased with decreasing habitat suitability (Fig. 2a). There was no such trend between survival rate and habitat suitability (Fig. 2b).

Table 3. Summary of Generalized Linear Mixed Models (GLMMs) testing the effects of time, habitat suitability, disturbance treatment and seed addition density on the number of seedlings of *Campanula thyrsoides* in a seed sowing experiment

Source of variation	Model effects	
	df	Test statistics
Number of seedlings		
Time	2	580.03****
Habitat suitability	1	8.20*
Disturbance	1	3.31(*)
Seed density	2	46.09****
Contrasts low vs. medium		-4.87****
Contrasts medium vs. high		11.42****
Disturbance:Seed density	2	0.71
Site	1	27.52****

df, degrees of freedom. Test statistics are F-values for fixed effects (*Habitat suitability*, *Disturbance*, *Seed density*, *Disturbance:Seed density*), t-values for *a priori* contrasts, and ChiSquare values for random effects (*Plot, Site*). *Time* represents a factor of three different censuses of measurements. Habitat suitability was measured by the Beals index (for details see Materials and methods). Plots (n = 270) are nested in sites. Asterisks represent significance levels: (*)$P < 0.08$; *$P < 0.05$; ****$P < 0.0001$.

Time had a much stronger negative effect on the number of seedlings than habitat suitability (Table 3). Seedling diameter did not differ among sites of different occupancy and habitat suitability (Tables 2 and 4).

Table 4. Means (SE) for germination rate, seedling diameter and survival rates in a seed sowing experiment with *Campanula thyrosides*. Means were calculated for the habitat quality, seed addition density and disturbance treatment separately

	Habitat quality		
	low	medium	high
Germination, %	1.22 (0.3)	1.36 (0.5)	0.18 (0.1)
Diameter, mm	5.9 (0.4)	4.0 (0.3)	5.4 (0.4)
Survival 2009, %	32.85 (5.4)	31.53 (6.5)	39.92 (7.5)
Survival 2010, %	12.94 (3.0)	13.21 (4.5)	17.40 (6.0)
	Seed density		
	300	3,000	30,000
Germination, %	1.18 (0.4)	0.77 (0.3)	0.54 (0.1)
Diameter, mm	5.1 (0.6)	4.6 (0.3)	5.6 (0.3)
Survival 2009, %	27.81 (9.4)	31.76 (6.1)	39.96 (4.6)
Survival 2010, %	10.42 (5.7)	13.54 (4.1)	16.84 (3.4)
	Disturbance		
	undisturbed	disturbed	
Germination, %	0.73 (0.2)	0.93 (0.3)	
Diameter, mm	4.5 (0.3)	5.8 (0.3)	
Survival 2009, %	26.59 (4.6)	41.07 (5.3)	
Survival 2010, %	8.00 (2.4)	19.52 (3.8)	

Habitat quality: low = unoccupied and unsuitable sites; medium = unoccupied, but suitable sites; high = occupied and suitable sites for *C. thyrsoides*. Suitability of sites was measured by the Beals index (for details see Materials and methods). Seed density, number of added seeds. *n* = 90 plots.

Finally, the habitat suitability correlated positively with slope, soil pH, rock cover and cover of dwarf shrubs, and negatively with grass cover (Table 5).

Table 5. Correlation analysis of habitat suitability with characteristics of 15 experimental sites in a seed sowing experiment with *Campanula thyrsoides*

	r
Slope (%)	0.84****
Soil pH	0.70**
Species richness	-0.20
Rock cover (%)	0.61*
Grass cover (%)	-0.62*
Cover of Fabaceae (%)	-0.13
Cover of dwarf shrubs (%)	0.66**

For details to the habitat suitability measured by the Beals index see Materials and methods. *r*, Pearson's correlation coefficient; Species richness, number of species assessed in vegetation relevées. $n = 15$. Asterisks represent significance levels: *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

Effects of disturbance

Disturbance had the strongest positive effect on all measured variables, with the exception of the number of seedlings (Tables 2 and 3). In disturbed plots, germination rate, survival rates, diameter of seedlings and the number of seedlings were always higher compared to undisturbed plots (Table 4; Figs 3 and 4). The disturbance treatment also had a positive effect on the number of seedlings in control plots, with on average 2.2 seedlings in undisturbed plots and 8.0 seedlings in disturbed plots.

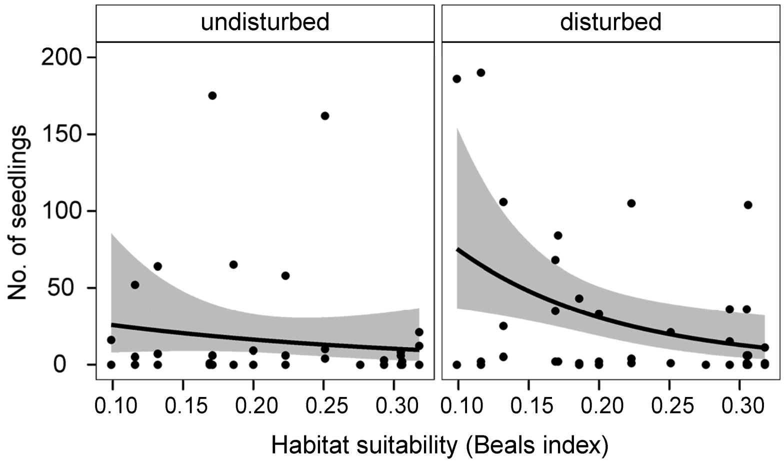


Fig. 3. The number of seedlings in 2009 in a seed sowing experiment with *Campanula thyrsoides* as a function of disturbance treatment and habitat suitability (measured by the Beals index; for details see Materials and methods). The lines represent the fit obtained by the model analyses and the grey shaded areas are the 95% confidence intervals.

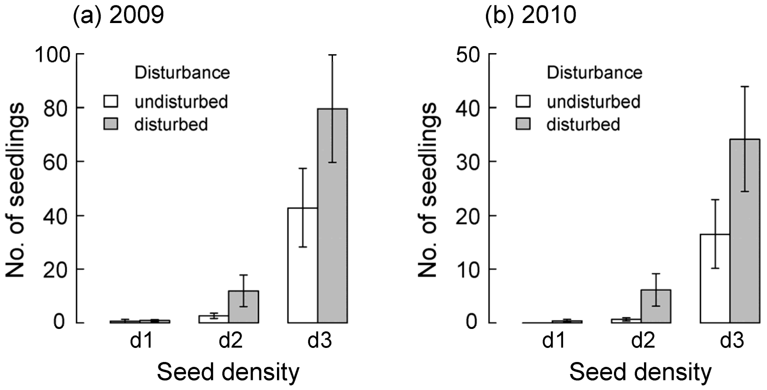


Fig. 4. The effects of seed addition density and disturbance treatment on the number of seedlings in **a** 2009 and **b** 2010 in a seed sowing experiment with *Campanula thyrsoides*. Seed density: d1, 300 seeds; d2, 3,000 seeds; d3, 30,000 seeds.

Effects of seed density

The number of added seeds significantly affected germination rate, seedling diameter and the number of seedlings, but not survival rates (Tables 2 and 3). The number of seedlings increased with increasing seed density (Fig. 4). Contrast tests indicated that the number of seedlings was different ($P < 0.0001$) between plots of low and medium seed density, and of medium and high seed density (Table 3).

The number of seedlings in control plots (mean = 5.1) was not significantly different from that observed in plots in which seeds were added in high density (mean = 9.0; $t = -1.14$, df = 11, $P = 0.28$). Moreover, the number of seedlings in control plots did not differ significantly from that in plots in which seeds were added in medium (mean = 1.2; $t = 1.13$, df = 11, $P = 0.28$) or low density (mean = 0.1; $t = 1.45$, df = 11, $P = 0.18$).

Discussion

Habitat suitability and seedling establishment

Germination rate and the number of seedlings of *C. thyrsoides* were significantly lower in experimental sites of high habitat suitability for adults measured by the Beals index. This negative relationship contradicts our hypothesis and results of another study with *Succisa pratensis*, where the Beals index correlated positively with the number of seedlings (Milden *et al.* 2006). In a long-term study that investigated the effects of habitat suitability on the establishment of seedlings in several grassland species, positive correlations between the Beals index and the presence of seedlings were generally weak and there was a trend of an increasing positive correlation over time (Ehrlén *et al.* 2006). It is likely that environmental conditions for successfully reproducing adults of *C. thyrsoides* are better in occupied compared to unoccupied sites, and that with time and increasing plant growth the number of plants would decrease in unoccupied and unsuitable sites (Gustafsson *et al.* 2002). However, within the two consecutive years of our experiment, the number of seedlings decreased in all sites similarly, and survival rates did not differ between sites of different habitat quality (Table 4).

Our results suggest that the Beals index is only valid for the habitat requirements of adult *C. thyrsoides*, based on which habitat suitability was assessed (Wüest 2008). The habitat suitability measured by the Beals index may not represent the suitability of a habitat for colonisation by seedlings, but rather the probability of a habitat to be occupied by an already established population. We know from integral projection models that populations of *C. thyrsoides* can moderately grow and persist even at an extremely low establishment rate of seedlings (Schynige Platte: seedling establishment rate < 0.1%; Kuss *et al.* 2008). Therefore, habitat conditions for successful recruitment in natural populations have most probably changed since colonisation due to the ongoing vegetation succession. We assume that *C. thyrsoides* needs open and newly created habitats to establish populations and that after a population has been established, it can persist for a long time, even when the habitat is changing. The correlation analysis supports that recruitment of natural populations may be negatively influenced by vegetation succession. Occupied sites had an increased cover of dwarf shrubs, while in unoccupied sites with lower suitability for *C. thyrsoides*, the vegetation was dominated by grasses (Tables 1 and 5).

The long persistence of *C. thyrsoides* at already occupied sites might be explained by a seed bank, which is known from other *Campanula* species (e.g. Greve Alsos *et al.* 2003), although we have no indication from literature or field for a persistent seed bank in *C. thyrsoides* (Hegi 1975; Kuss *et al.* 2007). A soil seed bank might increase persistence of populations in already occupied sites, but apparent colonisation of new unoccupied sites can hardly be due to a seed bank, because the area of the Schynige Platte has been monitored for *C. thyrsoides* since 2005. However, a seed bank would not change our interpretation that *C. thyrsoides* has a weak colonisation potential.

Limitation at the regional scale

Habitat limitation is not an important factor for the actual distribution of *C. thyrsoides* on the Schynige Platte, because only a small fraction (26%) of all suitable habitats was occupied by the species in recent vegetation relevées (Wüest 2008). Therefore, and because seeds lack morphological structures for wind or animal dispersal (Kuss *et al.* 2007), we hypothesised that dispersal limitation could be much more important than habitat limitation for the regional distribution of this species. Indeed, our seed addition experiment indicates that *C. thyrsoides* is strongly dispersal limited, since adding seeds to unoccupied habitats resulted in successful germination and survival of seedlings. In a seed sowing experiment with several plant species on a glacier foreland in the Eastern Alps, it was suggested that dispersal limitation might be a common feature of alpine plants (Erschbamer *et al.* 2008).

Nevertheless, recording seedling survival over more than two years, as was done in our study, would provide better estimates of dispersal limitation, since only long-term data could show whether seedling recruitment in unoccupied sites would lead to a sustainable population (Gustafsson *et al.* 2002). Although the survival rate of seedlings decreased drastically from the first to the second year (Table 4), we do not expect a significant decrease in the number of juveniles in the following years. This low mortality of plants in the near future is expected, because the early establishment is the most critical phase in the life cycle of many plant species (Cook 1979), including that of *C. thyrsoides*, because its survival probability increased with plant size in a demographic study (Kuss *et al.* 2008). Most of the young plants that survived the first winter in 2009 will probably grow into adults and will reproduce with an average flowering age of about 10 years (Kuss *et al.* 2008).

Limitation at the local scale

Our hypothesis that distribution of *C. thyrsoides* at the local scale is fully seed limited must be rejected, since augmentation of seeds in already occupied sites did not result in a higher number of seedlings compared to control plots. Consequently, an already established population of *C. thyrsoides* produces enough seeds to ensure its local spread and we must accept the alternative hypothesis that the species is partially microsite limited. Microsite limitation is supported by the result of the artificial disturbance. Disturbance increased the number of seedlings considerably (Figs 3 and 4), probably because ideal microsites facilitated germination of seedlings. Such microsites created by disturbance may include increased light availability and higher soil temperatures, which were measured in plots free from vegetation in another sowing experiment (Chambers *et al.* 1990). Interspecific competition was also reduced by our disturbance regime, but intraspecific competition could still be present in disturbed plots, which is assumed to be higher than competition between seedlings of different plant species (Svenning *et al.* 2008).

Intraspecific competition for nutrients might at least partially explain the low survival rate in the second year, which was nearly half of that observed in the first year (Table 4), when seedlings were smaller and their roots were less intermingled.

Furthermore, the average germination rate in permanent plots of a natural population of *C. thyrsoides* on the Schynige Platte was estimated to be 0.078% (Kuss *et al.* 2008), while in a greenhouse study germination on wet filter paper was ca. 1,000 times higher at 75% (Ægisdóttir *et al.* 2007). Thus, the low seedling recruitment in natural populations of *C. thyrsoides* is rather due to a lack of microsites than a limited availability of seeds. Indeed, when simulating population growth in *C. thyrsoides*, increasing the seedling establishment rates resulted in a dramatic increase of the population size (Kuss *et al.* 2008). Although we have no indication for seed limitation, seed predation could occasionally play a role for the local distribution of *C. thyrsoides* (Kuss *et al.* 2007), because pre-dispersal seed predation is a crucial factor affecting seed availability (Juenger & Bergelson 2000; Szentesi & Jermy 2003; Orrock *et al.* 2006).

Conservation implications

Summarising our results, the species *C. thyrsoides* may become endangered on the Schynige Platte in the future because of its weak colonisation potential in combination with its narrow ecological niche, monocarpy and strong self-incompatible breeding system known from previous studies (Ægisdóttir *et al.* 2007; Kuss *et al.* 2007; Wüest 2008). Although genetic diversity was generally high in all populations in this region (Frei *et al.* submitted), we would consider the currently established natural populations of *C. thyrsoides* as threatened, if the present availability of suitable habitats diminished because of global warming. Shifts in the distribution of alpine plants in response to increased summer temperatures have been repeatedly reported (Grabherr *et al.* 1994; Walther *et al.* 2002; Parmesan & Yohe 2003). The expected range extension of plants because of global warming is likely to be related to their different dispersal abilities (le Roux & McGeoch 2008), and we suggest that particularly dispersal limited species such as *C. thyrsoides* will be negatively affected by climate change.

A long, traditional management history is obviously important for the persistence of many plant species (Eriksson 1998) and for *C. thyrsoides* in particular, because suitable habitats are abundantly available on the Schynige Platte (Wüest 2008). Thus, another putative risk for the population persistence of *C. thyrsoides* might be changes in the current land use practises (Körner 2003). For several centuries and to this day, the area of the Schynige Platte has been used as summer pasture for cattle (Lüdi 1948). Pastures, which are more heterogeneous and characterized by light gaps in the vegetation from disturbances by grazing animals, favour the establishment of seedlings much more than homogeneous mown grassland does (Bullock *et al.* 1995; Coulson *et al.* 2001). Nonetheless, such disturbances by cattle are obviously not enough for the establishment of new populations at suitable unoccupied sites, especially since *C. thyrsoides* often grows on steep slopes where cattle is absent and where, as a result, dwarf shrubs dominate. An appropriate management strategy for conservation of the rare and strongly dispersal limited *C. thyrsoides* at the regional scale might therefore be to assist the dispersal of this species by sowing seeds from local plant populations in suitable habitats and to prevent succession by a sustainable land use management.

At the local scale, a disturbance regime would be worthwhile during the first years after sowing, which has been shown to increase seedling establishment in *C. thyrsoides* (Table 2), as well as in other grassland species (Klinkhamer & De Jong 1988). Such artificially disturbed sites are best suited for germination and early survival in *C. thyrsoides*; over 180 seedlings m^{-2} were growing in such a site (Furka Pass, author's personal observation).

Conclusions

Our results suggest that habitat suitability based on the Beals index for established populations of *C. thyrsoides* is not well suited to determine whether a habitat is also suitable for germination and successful establishment of seedlings in this rare alpine plant. Disturbance affected the number of seedlings positively, indicating the importance of vegetation gaps for the abundance of *C. thyrsoides*. The strong dispersal and microsite limitation of *C. thyrsoides* as well as the succession in sites where populations established long ago may endanger this species on the

Schynige Platte and in other regions in the Alps. Rare plant species including *C. thyrsoides* with a weak colonisation potential will be disadvantaged if habitats continue to shrink and no species-specific management strategies are implemented, as we propose in the present study.

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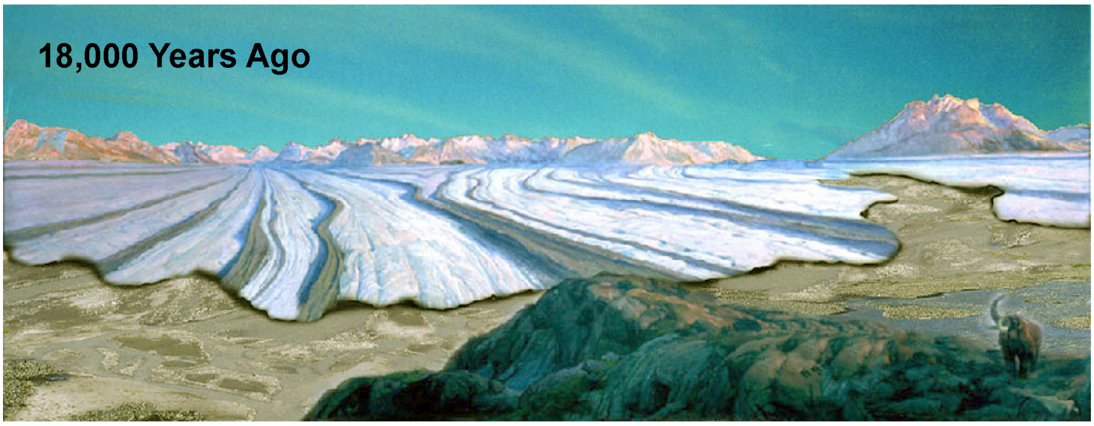
Chapter 7

General summary and discussion



The focus of this thesis lies on the phenotypic population differentiation (Chapters 2 & 3) as well as on the genetic structure and gene flow dynamics (Chapters 4–6) in Alpine plants. These topics were addressed by a variety of different methods in the laboratory and field, and by investigation of population processes at different spatial scales in the European Alps. This chapter summarises the most important results of the five preceding chapters and discusses the significance of our findings in the larger context of historical biogeography, landscape genetics and implications for conservation. The new basic knowledge gained from this work is also a starting point for extending research questions in the field of plant population ecology, and therefore, priorities for future research are proposed. The final section highlights the main conclusions.

The impact of glacial history on large-scale plant differentiation

The genetic diversity within and among populations of a plant species is affected by several factors as outlined in Chapter 1. There is a long-running debate about the relative importance of species-specific life-history traits, glacial history and ecological factors affecting the spatial genetic structure in plants (Hamrick & Godt 1996; Cox & Moore 2005). In the first part of this work (Chapters 2 & 3), we have used both molecular and experimental methods to study the consequences of glacial history and environmental variation on regional genetic structure and phenotypic differentiation in the two Alpine plants *Geum reptans* and *Campanula thyrsoides* at the large scale of the Alps.

G. reptans probably survived the last Ice Age in four peripheral refugia at the border of the Western, Central-Western, Central-Eastern, and Eastern Alps (Alvarez *et al.* 2009). In our study, plants of 16 populations of *G. reptans* were sampled from the Alps and analysed with molecular markers. With the molecular analysis, evidence for four spatially separated genetic groups was found, i.e. West, Central-West, Central-East and East Alpine groups (Chapter 2). This regional molecular structure corresponds to the theory that the Alps were recolonised by several phylogeographic lineages which have been differentiated due to neutral genetic drift and limited gene flow during isolation in the proposed glacial refugia. However, the Central Alpine group was not well separated in a western and eastern group, and thus it was concluded that contemporary gene flow is quite substantial among the two Central Alpine groups and is therefore already masking the effect of recolonisation out of different refugia.

In the common garden experiment, the phylogeographic structure of *G. reptans* was reflected by strong regional differentiation in growth, reproduction and leaf morphology, as well as in growth response to competition. Similar to *G. reptans*, regional differentiation in morphological and phenological traits of *C. thyrsoides* in the common garden was found (Chapter 3). Populations from the Southeastern Alps showed a delayed and indeterminate flowering compared to populations from the other phylogeographic regions. Additionally, in populations from the Western Alps a lower susceptibility to grazing was observed, maybe related to their predominant occurrence in pastures and meadows in that region.

In summary, neutral genetic processes during glacial history not only affected genotypes, but also phenotypes in both plant species. The results clearly demonstrate the importance of Quaternary history for the phylogeographic structure and phenotypic differentiation in Alpine plants. Nevertheless, we cannot completely rule out that one of the following factors contributed to the observed population differentiation in *G. reptans* and *C. thyrsoides*:

(i) Adaptive processes to historical (Hewitt 1996) or recent environmental conditions (Becker *et al.* 2006) – Comparison of the quantitative trait differentiation (Q_{ST}) with neutral molecular differentiation (G_{ST}) suggests that selection has contributed to phenotypic differentiation in most traits of *G. reptans*. Significant correlations of traits with climatic variables at the original sites of the populations indicated that adaptation has affected differentiation in growth of *G. reptans* (Chapter 2). In *C. thyrsoides* it is most likely that the mentioned differences in phenology are due to an adaptation of the plants from the Southeastern Alps to the recent submediterranean climate (Chapter 3). Therefore, the relatively high adaptive potential detected in both Alpine plant species is partially related to differences in the recent climate at the sites of population origin.

(ii) Recent neutral genetic processes – Limited gene flow due to pronounced landscape structures among recolonised phylogeographic regions (e.g. Aosta valley between the Western and Central Alps) may have contributed to genetic differentiation in both species.

(iii) Species-specific biology (Nybom 2004) – It has been suggested that wind-dispersed Alpine plant species such as *G. reptans* have a higher genetic diversity than species with other dispersal modes (Thiel-Egenter *et al.* 2009). However, we found a rather low genetic diversity in this species (Chapter 2). Therefore, effects of the species' biology could be masked by effects of glacial history, i.e. small population sizes and founder effects (Nei *et al.* 1975) during recolonisation.

Plant differentiation and distribution at small spatial scales

In Chapters 2 & 3, we considered the glacial history and the recent climate as major determinants for Alpine-wide population differentiation in Alpine plants. However, at the smaller regional scale of populations on a mountain plateau in the Swiss Alps (Chapter 4) or even at the local scale of a single population (Chapter 5), landscape structure and species-specific traits gain relative importance for population differentiation, because all populations in a small region are likely to have the same phylogeographic origin. Spatial isolation of populations due to a highly structured landscape is an important factor for the genetic structure of populations, since limited gene flow between plant populations may enhance differentiation. But gene flow also strongly depends on species-specific traits such as life-history (Vitalis *et al.* 2004), breeding system (Ægisdóttir *et al.* 2007), dispersal mode (Thiel-Egenter *et al.* 2009) and phenology (Hirao & Kudo 2004).

In the second part of this doctoral thesis (Chapters 4–6), we have investigated small-scale population differentiation and gene flow dynamics, as well as the seed dispersal and colonisation potential of the Alpine plant species *Campanula thyrsoides* in the Swiss Alps with molecular tools and field experiments.

As compared to differentiation observed at larger scales in the Alps, a considerably high genetic diversity was found both within ($H_e = 0.71$) and among ($G'_{ST} = 0.32$) all 24 populations of *C. thyrsoides* existing on the Schynige Platte within an area of approximately 10 km^2 (Chapter 4). The absence of isolation-by-distance suggests that drift caused by spatial isolation of small populations is the more influential evolutionary force in shaping the genetic structure of *C. thyrsoides* than gene flow by seeds, which is restricted due to the lack of morphological structures to support dispersal.

Four-year monitoring data showed that only a small fraction of all individuals in populations of *C. thyrsoides* flowers in one specific year. Therefore, the limited mating possibilites due to the monocarpy of *C. thyrsoides* may additionally contribute to the substantial differentiation among populations on the Schynige Platte (Chapter 4) despite the relatively high gene flow by pollen (7.6%) into one single population (Chapter 5).

Based on the results of the seed sowing experiment with *C. thyrsoides* (Chapter 6), we suggest that the rareness of this monocarpic species on the Schynige Platte and presumably also in other Alpine regions is highly related to its limited seed dispersal at the regional scale and the absence of ideal microsites for successful germination of seedlings at the local scale.

Conservation implications

Several of the findings summarised above are important for the prediction of the consequences of environmental changes on the Alpine plant biodiversity in general, and for conservation management of the rare *C. thyrsoides* in particular.

Alpine plant biodiversity in a changing world

The results of this thesis show the importance of past climate dynamics and glaciations on the spatial genetic structure of two Alpine plant species (Chapters 1 & 2), indicating that plants have migrated into more suitable habitats in the forelands of the Alps during glaciations (i.e. *tabula rasa* hypothesis; Gugerli & Holderegger 2001) and remigrated into the Alps after the last Ice Age. These historical migration patterns are still imprinted in the genetic structure of plants, suggesting that species may respond by migration also to recent climate change (Huntley 1991; Hewitt 2004). Indeed, there is strong evidence that under the predicted climate change scenarios species will migrate to higher elevation in the Alps following favourable climatic conditions (e.g. Walther *et al.* 2002). Therefore, species range changes of similar extent as during the ice ages can be expected.

To this day, its not clear whether Alpine plant species that have already reached the summits of mountains and are not able to move higher will become extinct, and whether the Alpine plant richness will decrease within the next decades. However, in our studies (Chapters 1 & 2), both species showed indications for adaptation to variation in the recent climate and thus Alpine plants may respond to future climate change not only by migration but also by adaptation to new climatic conditions.

Management strategies for Campanula thyrsoides

The molecular results (Chapters 4 & 5) for *C. thyrsoides* suggest that gene flow is strong enough to overcome negative founder effects (i.e. low diversity and inbreeding depression; Ellstrand & Elam 1993) in the small and spatially structured populations of *C. thyrsoides* on the mountain plateau of the Schynige Platte. The high genetic diversity suggests that the species is not immediately at risk of extinction, which is also supported by a demographic study (Kuss *et al.* 2008).

Nevertheless, this does not mean that the susceptibility to extinction of *C. thyrsoides* is small, since any decrease in population size due to habitat loss could lead to an ‘extinction vortex’ as described in Chapter 1, particularly since *C. thyrsoides* has a weak colonisation potential (Chapter 6). Grasslands on the mountain plateau have been used as summer pasture for cattle to this day. An abandonment of this land use practice, for example due to socio-economical reasons, as occurred in other regions in the Swiss Alps (Rudmann-Maurer *et al.* 2008), would lead to habitat loss as a consequence of vegetation succession and immediately threaten the vulnerable species *C. thyrsoides*, which needs moderately disturbed sites for successful seedling establishment (Chapter 6).

In order to assure the long-term survival of *C. thyrsoides* on the Schynige Platte, assisting seed dispersal would be one efficient management strategy, since *C. thyrsoides* is strongly dispersal limited on this mountain plateau (Chapter 6). After sowing seeds into suitable but unoccupied habitats or within already established natural populations, it might be valuable to facilitate seedling establishment in *C. thyrsoides* by artificial disturbance, because the disturbance treatment considerably enhanced germination and seedling survival in this species (Chapter 6).

Priorities for future research

This chapter gives an overview of priorities for future Alpine plant research. The presented ideas emerged during the writing process of this doctoral thesis and are based on its approaches and results.

Our novel approach, the use of phylogeographical methods in combination with common garden experiments, may serve as reference for future studies to reveal whether it is a common feature in Alpine plants that glacial history and post-glacial recolonisation are reflected in adaptive genetic differentiation. Additionally, it has been shown that well established populations harbour more genetic diversity than recently founded populations which are impoverished genetically as a result of genetic bottlenecks (e.g. Mraz *et al.* 2007). In order to investigate whether the adaptive potential is also lower in recently founded populations, it would be promising to set up a common garden experiment with plant material from several populations originating from only one phylogeographic region (i.e. plants that recolonised the Alps out of the same glacial refugium). The results of such a common garden experiment together with a high resolution molecular analysis could provide detailed ecological information on the recolonisation pathways in both phenotypes and genotypes.

It is assumed that dispersal limited species have both, a weak migration potential (le Roux & McGeoch 2008) and a low genetic diversity (Thiel-Egenter *et al.* 2009), which may disadavantage them to either migrate or adapt in response to climate change. Therefore, it seems straightforward to conduct a large-scale study using more than two Alpine plant species with different dispersal modes, in order to compare their adaptive potential in response to global warming. To simulate the expected temperature rise, a field-based reciprocal transplant experiment (sensu Kawecki & Ebert 2004) along an altitudinal gradient could be used to study the long-term adaptive potential. Additionally, a climate chamber experiment with different temperature regimes could be performed to investigate the short-term response of plants (i.e. phenotypic plasticity) to elevated temperature. Results of these combined methods would give deeper insights in the question whether conservation measures for dispersal limited species should be prioritised, since they are likely to be losers of climate change. Furthermore, such a study would allow to estimate the relative importance of phenotypic plasticity. Since rates of climate change are most often not in line with those of migration and adaptation of plant species (Neilson *et al.* 2005), it is likely that particularly the ability of plants to respond to environmental change in the short-term may buffer them from rapid climate change. Such a high plasticity would allow the persistence of Alpine plant species until long-term survival by migration or adaptation is assured.

General conclusions

The previous discussion highlighted the important role of the spatial scale for population differentiation in Alpine plant species. Our results substantiated that at the large scale of the European Alps, (post-)glacial history and climatic variation strongly affect genetic diversity and phenotypic differentiation among populations, while at the smaller scale of a mountain plateau in the Swiss Alps, landscape structure and dispersal mode are more relevant for shaping genetic diversity.

The phylogeographic studies combined with common garden experiments showed that glacial history, migration patterns and regional variation in the recent climate are imprinted in the genetic structure of the Alpine plants *G. reptans* and *C. thyrsoides*. Here, we especially emphasise the importance of neutral genetic processes for phenotypic differentiation in Alpine plants, as a consequence of survival in isolated glacial refugia and founder effects during recolonisation.

The molecular studies on gene flow dynamics in *C. thyrsoides* on the Schynige Platte revealed a relatively high genetic differentiation despite considerable gene flow by pollen, most likely as a consequence of limited gene flow by seeds and the monocarpic life-cyle of the species.

Finally, the seed sowing experiment with *C. thyrsoides* on the Schynige Platte supported our molecular findings that dispersal limitation is a key factor for population dynamics in this species. Although the populations studied are not immediately threatened, only with respect to the high within-population genetic diversity, the field monitoring of populations should be continued in the following years and land use practices in this region should periodically be reviewed, since any change in the traditional land use could endanger *C. thyrsoides* with its weak dispersal ability in this Alpine region.

The questions addressed in this doctoral thesis contributed to the understanding of evolutionary processes within plant species, and to our knowledge of genetic diversity and gene flow dynamics at different spatial scales in the European Alps. However, our findings provide more than just new basic ecological information. They illustrate the useful combination of molecular approaches with classical field experiments to elucidate the complex processes in plant populations. Furthermore, valuable information for predicting the consequences of climate change on Alpine plant biodiversity was provided and conservation implications for the rare *C. thyrsoides* were emphasised. Particularly in the near future, when the sensitive Alpine ecosystem is undergoing changes by modern land use practices and global warming, a scientific fundament is needed for the development of a more efficient conservation policy. Future conservation management should involve planning of sustainable land use practices and re-evaluate the fixed concept of protected Alpine areas as it exists today. One effective conservation strategy could be to connect several nature reserves in the Alps with corridors. Such an approach would be more dynamic, as it also accounts for the changing environments and expected range changes of Alpine plants in a temporal and spatial dimension.

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Drawings of *Geum reptans* and *Campanula thyrsoides* are modified from Hess *et al.* (1976–1980), Flora der Schweiz und angrenzender Gebiete.

We do not inherit the earth from
our ancestors, we borrow it from our children.

Ancient Indian Proverb