Phenotypic divergence in widespread plants: genetic drift, selection and plasticity

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A journey of a thousand miles starts with a single step – Lao Tzu, Tao Te Ching, Ch. 64

The conditions were too advantageous to refuse – J.F. Scheepens „paraphrasing“ from Johann Bernoulli's autobiography

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Was nur Wert hat in der jetzigen Welt, das hat ihn nicht and sich, seiner Natur nach, – die Natur ist immer wertlos: – sondern dem hat man einen Wert einmal gegeben, geschenkt, und wir waren diese Gebenden und Schenkenden! – Friedrich Nietzsche, Die fröhliche Wissenschaft, sec. 301

I am convinced that Natural Selection has been the main but not exclusive means of modification – Charles R. Darwin, The Origin of Species, p. 6

Contents

Chapter 1

General Introduction

General Introduction

Lectori salutem,

Before introducing the aims, questions, methods and outline of this thesis, I would like to present some background of the scientific field of plant population biology and to delimit the spatial and temporal context of this thesis. Thus, I will shortly explain the terms **phenotype**, **genotype**, **evolution**, **natural selection**, **adaptation**, **fitness**, **random genetic drift** and **phenotypic plasticity**. Subsequently, I will address the **European Alps and its vegetation**, and the **Quaternary climate oscillations** which the Alpine vegetation experienced[1].

A concise introduction to evolution

A **phenotype** is the instantiation of an organism, and phenotypic traits are any characteristics observable from this organism. The phenotype results from the organism's **genotype**, the environment, and their interaction[2]. The genotype is the organism's hereditary information, usually understood as the specific sequences of DNA carried by the organism, called the genome. The genome is replicated very accurately, but rare copying mistakes as well as spontaneous mutations may occur, which may then be inherited by the next generation.

Evolution is the heritable change over time in the phenotype of an organism (Darwin 1859). Evolution can be observed at different scales. Microevolution concerns evolutionary changes within or among populations at relatively short time scales, whereas macroevolution concerns the process of speciation at relatively long time scales. A prerequisite for evolution is the presence of variation in the phenotype coded by the genotype. Particular variants in the population can be selected for by a process called **natural selection**, which eliminates those individuals maladapted to the environment. Paraphrasing Charles Darwin, natural selection is the „principle, by which each slight variation, if useful, is preserved". Therefore, natural selection is coupled to **adaptation** of populations to their environment. Those individuals that survive and produce more offspring than others are said to have higher **fitness**.

Evolution can also be neutral with respect to selection; the term **random genetic drift** is used for heritable changes in the population which are the result of chance effects (e.g. through limited population size; Conner and Hartl 2004). It is interesting that the importance of random genetic drift was long ignored. From Charles Darwin's correspondence, it is known that he understood the concept but hesitated to speak about it since his alternative theory to creationism was already quite bold in his days; the audience would not accept a notion of chance beside natural selection. In the 1920s, Sewall Wright, Ronald A. Fisher and J.B.S. Haldane laid the foundations for population genetics, a precursor for the modern evolutionary synthesis in the 1930s and quantitative genetics in the 1950s. It was Wright who developed the modern concept of random genetic drift, but it was fiercely debated whether random genetic drift played a minor (Fisher 1930) or a major (Wright 1948) role in evolution. Motoo Kimura (1968) reinvigorated the debate in the 1960s and it has since become a legitimate question to what extent trait differentiation is due to random genetic drift or adaptation.

We have read above that the phenotype is partly affected by the genotype, but we have not spoken about the role of the environment in shaping the phenotype. Imagine two plants, one in the sun, the other in the shade. All other resources being equal, the plant in the sun is likely to grow faster. In this scenario both plants acquire different phenotypes and this species can therefore

[1] Note that *Alpine* refers to the European Alps whereas *alpine* refers to the alpine life zone (Körner 2003). The terms Alpine flora and alpine flora therefore constitute two partly overlapping sets of species.

[2] For a different view on what constitutes the information-bearer of living organisms, see Cameron (2001), Luisi (2003) and Etxeberria (2004).

respond plastically to the environment. However, this response to the plants' environments is passive. In contrast, if the plants could somehow influence their own phenotypic destiny depending on a given environment, we call this **phenotypic plasticity** (Bradshaw 1965; Schlichting 1986)[3]. For example, the shaded plant could respond by growing long stem internodes to increase its chance to reach a sunny spot. Phenotypic plasticity is thus the ability of a genotype to adjust its phenotype depending on the environment. It is important to remember that this ability has itself a genetic basis.

To conclude, neutral genetic drift, natural selection and phenotypic plasticity are three not mutually-exclusive phenomena which can mechanistically explain morphological variability observed among individuals, populations or species of living organisms. These three phenomena are not mutually exclusive since observed differences could be the sum of the effects of more than one of these three processes. These phenomena are mechanistically explainable in so far that the scientific fields of population genetics and quantitative genetics provide the mechanistic theory to understand what is physically occurring at the level of the phenotype-genotype-environment interaction. In turn, these fields of study partly rely on and are partly explained by established knowledge from the fields of molecular biology and biochemistry.

This thesis explores neutral genetic drift, natural selection and phenotypic plasticity in plants, mainly in the setting of the European Alps. Since the European Alps are characterised by strong spatial heterogeneity in environmetal conditions (Körner 2003), selection pressures vary tremendously, demanding variable adaptations from Alpine species (Stöcklin et al. 2009). Moreover, environmental conditions are highly variable in time, not only throughout the seasons (Körner 2003) but also at time scales at which global climate change comes into play (Brockmann-Jerosch 1908; Schönswetter et al. 2005). A strong driver for phenotypic change were the Ice Ages, and their effects on phenotypic differentation of Alpine plants is a central topic of this thesis. In the following, I will give a general introduction to the Ice Ages, followed by an account of the effects of the Ice Ages on the Alpine flora.

The Ice Ages

A recurring hypothesis in this thesis is that glacial history affected the intraspecific evolution of widespread Alpine plants. Planet Earth has been subject to strong climatic changes ever since it came into existence 4.5 billion years ago (Kroonenberg 2006). Focussing on the colder periods, the first ice caps on the poles were formed in the middle of the Precambrian around 2.4 billion years ago (Webb and Bartlein 1992). After a long and warm intermezzo, much of the Earth became cyclically glaciated during the late Precambrian (c. 950 to 615 m years ago; Frakes 1979). Jumping forward in time, the Tertiary started with high temperatures across the globe and the ice caps were absent, but temperatures slowly cooled down again (Frakes 1979; Lockwood 1985). Through this cooling, the continental ice shelves started to grow around 3 million years ago. At least during the last million years of the Quaternary but possibly from earlier times on, the so-called Quaternary climate oscillations occurred with cycles of roughly 100,000 years. Six Ice Ages have been recognised from this period, named Biber, Donau, Günz, Mindel, Riss and Würm. During such a cycle ice accumulated rather slowly for 90,000 to 100,000 years but terminated quickly within about 10,000 years (Lockwood 1985). Relatively warm interglacials of about 10,000 years occurred in between these long Ice Ages. Records indicate that periods of cooling grew in intensity over time, with those true Ice Ages in the late Quaternary being much more extreme than those global coolings in the late Tertiary and early Quaternary (Lockwood 1985; Webb and Bartlein 1992).

Three processes in combination with geographic features make possible these drastic climate shifts, joined together in Milankovitch' theory (Milankovitch 1930). First, there is the orbital eccentricity of the earth around the sun (Hays et al. 1976) which causes major changes in insolation over a timespan of 100,000 years. Second,

[3]It is scientifically and philosophically very difficult to demarcate passive from active responses in living organisms. The central concept here is that of intentionality (Cameron 2001).

the axial tilt (obliquity) has a cycle of 40,000 years. In combination with precession (longitude of the perihelion), which cycle revolves every 23,000 years, these three processes produce a complex of climatic oscillations. But it is only in combination with tectonic movements, which forces wind and ocean currents in particular directions, that these oscillations can form such extreme climate changes called Ice Ages (Hewitt 1996).
The last full Ice Age condition was 20,000 years BP, which was the last glacial maximum of the Würm Ice Age cycle which started 135,000 years BP (Webb and Bartlein 1992; Hewitt 1996). The onset of the present interglacial, called the Holocene, was 18,000 years BP. Strong temperature changes of 10-12 °C within 5-10 years were not uncommon during the end of this last Ice Age. Lesser orbital cycles continued to affect the global climate, causing for instance a cold period named the Younger Dryas during the interglacial (a 1,000 years period around 10,500 years BP). Though warmer conditions than today prevailed from 8,000-6,000 years BP (Atlantic Period), the last 6,000 years experienced a relatively constant climate comparable to current climate (Lockwood 1985).
During an Ice Age, the thickness of the land-based ice sheet could be up to 3 km (Lockwood 1985). In Europe the ice sheet of the last Ice Age extended down to Cologne, and European mountain ranges were likewise covered by a thick ice sheet. These mountain ranges included those of Cantabria, the Pyrenees, the Alps, the Carpathians and the Caucasus Mountains. In between the Northern ice sheet and these ice-covered mountain ranges a tundra and cold steppe dominated due to strongly decreased rainfall and increased continentality (Schmitt 2007).

The Alpine flora and the influence of Ice Ages

Most of the work in this thesis is related to plant life in the European Alps. The questions and hypotheses considered in this thesis mainly focus on specific case studies of Alpine plants, since the European Alps are ideal to investigate processes and phenomena such as neutral genetic drift, natural selection and phenotypic plasticity. After an overview of the Alpine flora, the effects of Ice Ages on the mentioned processses are addressed in this section.
The Alps measure 900 km west-east and 500 km north-south and are the largest mountain system in Europe. Its geological, climatological and biogeographical complexity is vast (Ozenda 1988). The Alpine flora is highly diverse compared to the lowland. The Alps harbour 501 endemics out of c. 4,000 species, mostly distributed in the southern part of the Alps. This amounts to 7-8% of the Alpine flora against 3% in the European flora, and is higher as in other European mountain systems. Endemics occur on calcareous soils rather than on siliceous soils, probably because of the more heterogeneous spatial distribution of calcareous soils.
The overall immense floral diversity of the European Alps is often explained with the landscape heterogeneity, but Ice Ages are thought to play a significant role as well (Tribsch 2004; see below). As stated earlier, the European Alps are characterised by pronounced spatial heterogeneity in abiotic conditions and strong temporal dynamics (Körner 2003). Temperatures decrease, precipitation increases, the vegetation period is shortened and the frequency of extreme weather events increases with increasing altitude. Therefore, the European Alps are ideal to study adaptation to such environmental factors related to altitude. Field sites differing in, for instance, 5.5°C are positioned at only 1,000 m altitudinal difference, which offers relatively easy access. For the latitudinal variant of a similar study, one needs to travel 1,000 km between field sites for the same temperature difference.
Alpine plant life is seriously challenged by the environmental conditions in the landscape, and the Alpine flora is strikingly different from that of the lowland (Chapin and Körner 1995), harbouring fewer annuals and more clonal species (Stöcklin 1992; Körner 2003). Although environmental heterogeneity in the European Alps is practical from a scientist's point of view studying adaptation, it cannot be a substitute for the situation in the lowland. For instance, the dispersal ability of species may be comparable between mountains and lowland, but required dispersal distances to track climate change are much larger in latitudinal direction in the lowland than in altitudinal direction along moun-

tain slopes (Körner 2003). Also, compared to the lowland, the habitat heterogeneity of the European Alps has different genetic consequences as populations are naturally small and isolated from each other. Due to habitat fragmentation, lowland species currently face the same situation regarding the increasing isolation of populations, but they have not had time to adapt to surviving as small and isolated populations, which may have disastrous effects on their future survival (Young et al. 1996; Kuss et al. 2008).

Tracking the European Alps through time, the Alps formed during the Tertiary between 65 million years until 2 million years ago. During the Miocene (23.0-5.3 million years ago), the vegetation in Europe was largely subtropical. With the cooling during the Pliocene (5.3-2.6 million years ago) the vegetation became temperate in character. Over these millions of years, a particular Alpine vegetation could develop before the cycles of Ice Ages started c. 1 million years ago. Ozenda (1988) reports uncited work by Jerosch (1903?) that Pliocene cooling was slow enough for species to adapt to the new temperate conditions, and 15% of the current Alpine flora could be derived locally during this time.
In the face of climate change, species can either (i) migrate to track the climate belt they are adapted to, (ii) adapt to changing conditions in their current habitat, or (iii) go extinct. The Ice Ages caused big shifts in the distribution of species, but mountain systems and, for instance, the Mediterranean Sea formed strong barriers to dispersal. Mountain species, however, could track the climate changes more easily by descending the mountain slopes toward the lowland in times of extending ice sheets and by ascending them again when the ice retreated.
The first Ice Ages likely drove most thermophile Alpine species to extinction, but temperate species survived mainly in so-called glacial refugia outside of the Alps or on the occasional nunatak (i.e. a steep ice-free mountain slope; Stehlik 2000). North of the Alps, precipitation decreased dramatically during Ice Ages and the vegetation rapidly changed accordingly. Thus, many Alpine species could only survive in small areas north of the Alps, where precipitation was still sufficient for them (Schmitt 2007). Recolonisation of the Alps after the Ice Ages occurred from these glacial refugia and nunataks as well as from the southern European peninsulae (Ozenda 1988). After the ice started to retreat around 18,000 years BP, pollen records show that the vegetation tracked the changes in climate, also in the opposite direction during the cold period of the Younger Dryas. Pollen records indicate that around 6,000 years BP the vegetation distribution was largely similar to the present (Hewitt 1996).

The changes in the distribution of species induced by climatic oscillations has severe genetic effects. Rapid expansion by long-distance dispersal might cause genetic bottlenecks, reducing genetic diversity in colonising populations. The bottlenecked colonisers will dominate the genome, because later migrants can only contribute little to the established leading populations, since the rate of reproduction of the later arrivals is logistically low compared to the colonising populations which experienced exponential growth (Hewitt 1996 p253 refs). In mountain systems, however, the distances to be travelled are much shorter than in the lowland as temperature change for each 1,000 m of difference in altitude equals 1,000 km of latitude (Ozenda 1988). Therefore, bottlenecking and consequent loss of allelic diversity may be weaker for mountain plants recolonising the mountains after ice retreat. Nevertheless, genetic studies commenced from the 1980s onwards do show strong phylogeographic patterns and reduction of genetic diversity along recolonisation routes (Schönswetter et al. 2005; Parisod and Besnard 2007). Although specific phylogeographic signals of the effects of Ice Ages appeared in several species, and since specific environmental factors may have affected their spatial genetic structure (Alvarez et al. 2009), the complexity of interactions between environmental, ecological, genetic, historic and other factors ensures that „each species group will have its own detailed story which requires individual research and telling“ (Hewitt 1996).

The aim of this thesis

The key elements that are addressed in this thesis are threefold: (1) effects of neutral genetic drift, natural selection and phenotypic plasticity on phenotypic differentiation; (2) effects of glacial history, geography and climate on phenotypic differentiation and adaptation; (3) genetic structure and gene flow at small spatial scale. Combining all three elements, the aim of this thesis is to understand how a plant species' evolution towards its current state is affected at different spatial scales by neutral genetic drift and historical as well as more recent environmental influences.

Main research questions

The central question of this thesis is *whether glacial history affected phenotypic differentiation in widespread alpine plants*. The follow-up question is *whether any such glacial history-related phenotypic differentiation is the result of adaptation or neutral genetic drift*. To address these two questions, largely considered in **Chapters 4, 5, 6, 10 and 11**, we used the widespread Alpine plant species *Campanula thyrsoides*, *C. barbata* and *Geum reptans*. From earlier work we already have good knowledge on the distribution, ecology and population genetics of *C. thyrsoides* and *G. reptans*. In order to assess effects of glacial history on phenotypic differentiation, it is necessary to understand patterns of glacial survival and recolonisation. For *Campanula thyrsoides* and *G. reptans* we performed molecular analysis to gain insight in their past (**Ch. 3**).

Effects of glacial history on widespread alpine plants are ideal to investigate the dichotomy of adaptation versus neutral genetic drift, since stochastic effects may have played a large role during glaciations, especially while migrating out of and back into the Alps. But evolutionary forces can be divided into another dichotomy: diversifying selection occurs when different trait means are selected for, whereas unifying selection occurs when the same trait mean is selected for among two or more populations. I have researched the possibility for either neutral processes or diversifying or unifying selection in various traits in two lowland species *Scabiosa columbaria* (**Ch. 2**) and *Campanula rotundifolia* (**Ch. 12**). In small regions, such as in the study with *S. columbaria*, unifying selection could be hypothesised since environmental conditions are similar across populations, whereas over large investigated transects, as in the study with *C. rotundifolia*, one could expect diversifying selection due to adaptation to variable conditions.

Another important set of questions relates to a more local scale and considers gene flow among populations of *C. thyrsoides* and establishment rate (**Ch. 7, 8 and 9**). More specifically, these questions are derived from former research indicating considerable population differentiation among populations and simultaneously high genetic diversity within populations. These observations may seem contradictory since high genetic diversity is usually associated with genetic connectivity, which should prevent among-population differentiation. Therefore, the main questions of this part of the thesis are to assess in a small region (i) *how seed and pollen dispersal and the resulting genetic diversity are structured in time and space*, (ii) *what this tells us about the colonisation history and future*, and (iii) *how the seeming contradiction between levels of within and among genetic diversity can be explained*.

Experimental approach

The most important tool used for the investigations reported in this thesis is the common garden experiment (**Ch. 2, 4, 5, 6, 10, 11, 12**), which is used to investigate genetically based phenotypic differentiation (Turesson 1922; Clausen et al. 1948). Seeds are sampled from different source populations and the produced offspring grown in a single environment. In the 1940s, Clausen, Keck and Hiesey (1948) conducted common garden experiments with alpine plants from the Californian mountains, establishing that the different ecotypes, i.e. varieties in the species' phenotype, were partially fixed irrespective of the location where they were grown, which was attributed to genetic factors. Since then, the method is still widely in use but the field of quantitative genetics has evolved drastically, allowing for instance precise analyses of heritability of traits.

In the 19th century Naegeli attempted to grow alpine plants in Munich and Kerner (1869) transplanted lowland plants to high elevations in Tyrol. Both transplantations were not very successful, leading Kerner to speculate about some kind of genetic adaptation. Bonnier (1890, 1895) transplanted plants reciprocally between the French Alps and the Pyrenees and concluded that the environment had the most influence on plant growth (Körner 2003). These studies were probably the first attempts to investigate the phenotypic plasticity of traits in different environments (**Ch. 6**). The inflexibility of various ecotypes studied by Clausen et al. (1948) has been attributed to local adaptation. Plants can be locally adapted to their environment since forces of natural selection often vary in space (Kawecki and Ebert 2004). Common garden experiments are ideal to investigate genetic differentiation in phenotypic traits among selected populations, but it cannot prove whether any observed differentiation is due to adaptation or neutral genetic drift. Reciprocal transplantation experiments, in which plants from different source populations are transplanted into each source population, can be used to prove local adaptation. In each population, plants transplanted in their home population should then have higher fitness compared to plants originating from other population (Kawecki and Ebert 2004). If such a pattern of local adaptation is not found, multiple alternatives are possible, such as (i) strong gene flow among populations precluding local adaptation or (ii) differentiation by neutral genetic drift. **Chapter 6** of this thesis presents an experiment which can be classified as halfway in between a common garden and a reciprocal transplantation experiment. By using three common gardens at different altitudes, phenotypic plasticity in traits to altitude can be investigated. Since source populations originate from a range of altitudes, adaptation of specific traits to „home“ versus „away“ conditions can be assessed with respect to these traits. However, it cannot be deduced whether the plants as a whole are adapted to the population of origin (Kawecki and Ebert 2004). The research project included reciprocal transplantation experiments, which have been conducted for *Campanula thyrsoides*, *C. barbata*, *C. rotundifolia* and *Geum reptans*, but the resulting data was either statistically not strong enough or has not yet been analysed.

Gene flow was mentioned as a force potentially precluding local adaptation (Kawecki and Ebert 2004). In fact, strong gene flow could even halt speciation. Gene flow is therefore a crucial factor in evolutionary biology. The amount of gene flow among populations can be estimated based on molecular differentiation, but this method is not very reliable. To assess the amount of gene flow into a population more accurately, paternity analysis can be conducted, which uses codominant molecular markers to infer parenthood of offspring (Ashley 2010). Paternity analysis is statistically and computationally heavy, but multiple computer programs are available nowadays (e.g. Kalinowksi et al. 2007). As an alternative to molecular methods, the use of fluorescent pollen to measure pollen dispersal has never been very popular, but its easy and cheap application may favour a revival (Van Rossum et al 2011). We use both methods to infer pollen movement within and into a single population of *Campanula thyrsoides* (**Ch. 8**).

Classical ecological experiments have not been forgotten. To investigate whether the local distribution of *Campanula thyrsoides* is limited by seed dispersal or by the availability of microsites, a seed sowing experiment was performed (**Ch. 9**). Germination and establishment of sown seeds would then indicate seed dispersal limitation, whereas failure to germinate and establish would indicate microsite limitation. Sowing locations were ranked by quality based on Beals index, which is based on species co-occurrence, in order to test the hypothesis that habitat of higher quality for *Campanula thyrsoides* would show the best results.

Molecular analysis of genetic variation is widely used for phylogeography, as in **Chapter 3** in which *Campanula thyrsoides* population from across the European Alps and the Jura Mountains were investigated to gain insight in its glacial history. Also at smaller scales, investigating molecular genetic variation of multiple populations may offer insight in current and past gene flow as well as historic bottlenecks and inbreeding (**Ch. 7**).

By comparing phenotypic differentiation with molecular differentiation, known as Q_{ST}-F_{ST} analysis, past selection pressures can be inferred

(**Ch. 2, 4, 6, 11,12**; McKay and Latta 2002). Molecular differentiation is assumed to be neutral and therefore presents some kind of background differentiation against which differentiation in phenotypic traits can be compared. If traits diverged less than expected based on this neutral differentiation ($Q_{ST} < F_{ST}$), past unifying selection among populations can be concluded. When phenotypic traits diverged more ($Q_{ST} > F_{ST}$), past diverging selection among populations can be concluded. If the two measures of differentiation do not differ statistically ($Q_{ST} = F_{ST}$), no selection pressure can be invoked (Spitze 1993; McKay and Latta 2002). Q_{ST}-F_{ST} analyses have conquered an important position in evolutionary biology, but the integrity of the theoretical basis is still debated (**Ch. 2**).

Outline of this thesis

Chapter 1 General Introduction – this chapter.

Chapter 2 Unifying selection acts on competitive ability and relative growth rate in *Scabiosa columbaria*

***J.F. Scheepens**, J. Stöcklin, A.R. Pluess*
Basic and Applied Ecology (2010) 11: 612–618

Competitive ability and relative growth rate are important fitness-related traits, especially in plants occurring in species-rich meadows, such as *Scabiosa columbaria*. It is therefore likely that these traits experience unifying selection, since any degradation in performance would be selected against, whereas favourable evolution in these complex polygenic traits is unlikely. Using existing genetic and phenotypic data on *S. columbaria*, we performed Q_{ST} versus F_{ST} comparisons to infer the existence and type of past selection pressures. We also discussed the validity of our conclusions, since the robustness of Q_{ST}–F_{ST} analysis is still debated.

Chapter 3 Spatial genetic structure of *Campanula thyrsoides* across the European Alps: indications for glaciation-driven allopatric subspeciation

*P. Kuss, G.F.J. Armbruster, H.H. Aegisdottir, **J.F. Scheepens**, J. Stöcklin*
Perspectives in Plant Ecology, Evolution and Systematics (2011) 13: 101–110

Glacial history may have had strong impact on intraspecific differentiation since species survived glaciations in multiple isolated refugia on the fringes of the European Alps. In this study, we investigated the neutral genetic differentiation among 51 populations of *Campanula thyrsoides* across the Alps and Jura Mts. using five microsatellite loci. The resulting phylogeographic clustering gives insight in the effects of glacial history on intraspecific differentiation and formed the basis for subsequent experiments on differentiation on the phenotypic level (**Ch. 4, 5 and 6**).

Chapter 4 Glacial history and adaptation explain regional differentiation in phenotypic traits in a widespread Alpine plant

***J.F. Scheepens**, E.S. Frei, J. Stöcklin*
Revised after review. To be submitted to *Ecology*

The aim of this study was to investigate whether the patterns of neutral genetic differentiation observed in **Chapter 3** were mirrored by differentiation in morphological

and phenological traits in *Campanula thyrsoides*. A common garden experiment was performed in which we grew offspring from 21 populations from across the European Alps and Jura Mts. In this way environmental effects on growth were eliminated so that any observed differences were due to genetic effects. A next step was to investigate if patterns of differentiation are due to genetic drift or due to adaptation to current or past selection pressures. Furthermore, three methods were applied which suggested adaptation of various traits: (i) explaining observed patterns in the common garden in terms of home-site advantage; (ii) correlation of phenotypic variability with environmental conditions at the locations of origin; (iii) Q_{ST} versus F_{ST} comparisons.

Chapter 5 Differentiation in morphology and flowering phenology between two *Campanula thyrsoides* L. subspecies

***J.F. Scheepens**, P. Kuss, J. Stöcklin*
Alpine Botany (2011) 121:37–47

Two subspecies of *Campanula thyrsoides* are recognised and their taxonomic status has been reconfirmed using genetic markers (**Ch. 2**). In this study we investigate in detail the morphological and phenological differentiation between the subspecies by analysing data from the same common garden experiment as in **Chapter 4**. The observed phenotypic divergence is explained by the differences in climate that the subspecies experience.

Chapter 6 Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to two different altitudes

***J.F. Scheepens**, E.S. Frei, J. Stöcklin*
Oecologia (2010) 164:141–150

Specific leaf area is a highly variable trait and strongly related to the biotic and abiotic environment. Interspecific variability in this trait is well investigated, whereas intraspecific variability has long been ignored. We performed a common garden experiment with *Campanula thyrsoides* using three gardens at contrasting elevations. This experiment enabled us to separate genetic from plastic effects and gave insight in the adjustability of this species to changing environmental conditions. The effect of phylogeographic origin of the species (**Ch. 2**) has also been taken into account in the analysis

Chapter 7 High genetic differentiation and founder effects in populations of an Alpine plant on a small and highly fragmented mountain plateau

*E.S. Frei, **J.F. Scheepens**, J. Stöcklin*
Submitted to *American Journal of Botany*

Former genetic studies on *Campanula thyrsoides* (*i.a.* **Ch. 2**) showed that populations harbour high genetic diversity but are also considerably differentiated from each other. This raises questions about (i) the genetic connectivity of populations and (ii) the genetic consequences of population establishment. We investigated 24 populations occurring within a small area (Schynige Platte) using five microsatellite loci to infer genetic differentiation and signals of founder effects.

Chapter 8 Monocarpic perenniality of *Campanula thyrsoides* results in high population differentiation despite high gene flow

***J.F. Scheepens**, E.S. Frei, G.F.J. Armbruster, J. Stöcklin*

Submitted to *Heredity*

Populations of *Campanula thyrsoides* harbour substantial among-population differentiation but also high within-population genetic diversity, even at local scale. This seeming contradiction begs the question to what extent gene flow is limited. We investigated pollen flow within and into a single population of the same group of populations on Schynige Platte as studied in **Chapter 7**. A paternity analysis was performed on 338 offspring sampled from the 22 flowering individuals of the study population in 2007. With molecular data from **Chapter 7**, we could assign any immigrants to populations outside of the study population. We discuss our results in the context of the monocarpic perenniality of *C. thyrsoides*.

Chapter 9 Limited colonization potential of a rare Alpine plant as extinction threat

*E.S. Frei, **J.F. Scheepens**, J. Stöcklin*

Submitted to *Plant Ecology*

The aim of this study was to assess the colonisation potential of *Campanula thyrsoides* in natural habitat. Additionally, it was investigated whether any constraint on colonisation potential was due to limitation in seed dispersal limitation or due to limitation in suitable habitat. Based on patterns of co-occurrence with other species, suitable and unsuitable habitat for *Campanula thyrsoides* was identified on Schynige Platte. Different amounts of seeds were applied on untreated as well as on deliberately disturbed soil, and the fate of seeds and seedlings was monitored over two years.

Chapter 10 Glacial history and adaptation explain differentiation in phenotypic traits in the Alpine grassland herb *Campanula barbata*

***J.F. Scheepens**, J. Stöcklin*

Submitted to *Plant Ecology and Diversity*

The hairy flowers of the herb *Campanula barbata* are a common sight in many subalpine meadows and pastures. A recent publication by Thiel-Egenter et al. (2011) provides locations of genetic breaks in the distribution of this species. To investigate whether the patterns of neutral genetic differentiation were mirrored by differentiation in morphological and phenological traits, and thus were likely due to glacial history, a common garden experiment was performed in which we grew offspring from 15 populations from two phylogeographic groups across a large part of the European Alps. To assess whether part of the trait differentiation is due to local adaptation, we performed correlations between trait values as observed in the common garden with environmental conditions of origin.

Chapter 11 Regional differences in growth, reproduction and leaf morphology mirror phylogeography of a widespread alpine plant

E.S. Frei, ***J.F. Scheepens****, G.F.J. Armbruster, J. Stöcklin*
Submitted to *Journal of Ecology*

Geum reptans is an alpine pioneer species from glacier forelands and is widespread in the European Alps, but populations occur strongly isolated. In order to investigate how glacial history affected neutral genetic and phenotypic variation, a molecular analysis was combined with a common garden experiment using 16 populations from across the Alps. Since *Geum reptans* is easily outcompeted by other species, a competition treatment was applied in the common garden. Growth, reproductive and leaf morphological traits were examined in this experiment. Furthermore, Q_{ST}–F_{ST} analysis was conducted to assess whether selection was at least partly responsible for any differentiation in phenotypic traits. In addition, correlations between phenotypic traits with altitude and climatic variables at populations of origin were conducted to investigate if adaptation to current local conditions may explain trait differentiation.

Chapter 12 Latitudinal and altitudinal differentiation in phenotypic traits and molecular markers of *Campanula rotundifolia*

V. Preite, J. Stöcklin, ***J.F. Scheepens***
In preparation for *Conservation Genetics*

Start of flowering and flowering duration are important fitness-related traits and intraspecific variation needs to be carefully adapted to latitude or altitude. We performed a common garden experiment with *Campanula rotundifolia* to investigate variation in these and additional traits along a latitudinal and altitudinal gradient. The study encompassed populations from Scandinavia, The Netherlands, and Switzerland, the latter including populations from the Jura Mts. as well as from the European Alps. Q_{ST} versus F_{ST} comparisons were conducted to investigate whether start of flowering, flowering duration and other traits had experienced selection in the past. Heritability of traits was assessed based on common garden data and gave insight in adaptative potential.

Chapter 13 General Discussion

Note Chapters 2, 3, 5 and 6 have been accepted in peer reviewed journals. The versions published in this thesis are the manuscripts as accepted by the respective journals. Minor changes have been or will be applied to these accepted versions for publishing by the respective journals.
Lay-out styles may differ among chapters, especially for the references, depending on the journal to which the manuscript has been submitted or in which the manuscript has been published.

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Chapter 2

Unifying selection acts on competitive ability and relative growth rate in *Scabiosa columbaria*

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Unifying selection acts on competitive ability and relative growth rate in *Scabiosa columbaria*

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Abstract

Q_{ST} vs. F_{ST} comparisons can reveal diversifying or unifying selection pressures among populations for specific traits. In this study we performed Q_{ST}-F_{ST} analyses on eleven populations of *Scabiosa columbaria* from the Swiss Jura to reveal genetic differentiation in two quantitative traits (above-ground biomass and relative growth rate of leaf lengths) and in neutral molecular markers. Above-ground biomass of plants under competition has been shown to correlate with their competitive ability, which is an important fitness-related trait. We hypothesized that strong unifying selection acts on above-ground biomass, since underperformance would result in decreased fitness and overperformance is unlikely due to trade-offs with other plant functions.
Overall G_{ST} (an F_{ST} analogue) was 0.12. Analysis of variance revealed that above-ground biomass and relative growth rate did not differ among populations, but both traits differed among seed families and were heritable (h^2=0.31 and h^2=0.35 respectively). Q_{ST} was close to zero for above-ground biomass and zero for relative growth rate of leaf lengths, and thus Q_{ST} was much lower than G_{ST}, indicating unifying selection on these traits.
This conclusion is restricted by the limits of the used methodology. $Q_{ST}<F_{ST}$ cannot always be considered as a proof for unifying selection, because in complex traits the assumption of purely additive effects of underlying genes may be violated. However, given the large differences between Q_{ST} and G_{ST}, together with substantial heritabilities of the traits under study, we conclude that our findings are not in contradiction with the hypothesis of unifying selection.

Keywords: RAPD; G_{ST}; above-ground biomass; Q_{ST} vs. F_{ST} comparison

Introduction

Natural selection is the driving force of evolution, causing adaptation. There is good evidence for adaptation in ecological traits to specific local conditions (e.g. Bradshaw 1984; Nagy and Rice 1997; Joshi, Schmid, Caldeira, Dimitrakopoulos, Good et al. 2001), but differentiation of ecological traits can also be due to neutral drift processes (e.g. Waldmann and Andersson 1998; Widén, Andersson, Rao and Widén 2002; Jorgensen, Richardson and Ander-

sson 2006). Q_{ST} vs. F_{ST} comparisons can discern between these two possibilities by comparing differentiation in quantitative traits (Q_{ST}) with differentiation in neutrally evolving genetic markers (F_{ST}). Selection can be either unifying or diversifying, i.e. quantitative trait values either remain constant or differentiate among populations, respectively. Thus, three possibilities arise: (1) under unifying selection, quantitative trait differentiation is selected against, but non-coding DNA, indicated by neutral genetic markers, will slowly but steadily differentiate if gene flow among populations is restricted, resulting in $Q_{ST}<F_{ST}$; (2) diversifying selection shifts quantitative trait means into opposing directions and the trait differentiation among populations would exceed differentiation of neutral genetic markers ($Q_{ST}>F_{ST}$); (3) under absence of selection pressures, quantitative traits are assumed to differentiate solely by drift and therefore at the same rate as neutral genetic markers ($Q_{ST}=F_{ST}$) (Rogers 1986; Spitze 1993; Merilä and Crnokrak 2001; McKay and Latta 2002).

The theory underlying the comparison of supposedly neutral markers with putatively non-neutral traits to infer selection can be traced back to Wright (1951). He showed that the additive genetic variance of a quantitative trait could be partitioned into within and among population components in the same mathematical framework as his F-statistics for neutral genetic markers. Some decades later, Spitze (1993) devised a statistic called Q_{ST} which quantified trait differentiation based on Wright's finding and which could be directly compared to F_{ST}. Ever since Spitze (1993) published the first comparison of quantitative trait differentiation with genetic marker differentiation to infer the presence and type of selection acting on different traits of *Daphnia obtusa* among eight populations, this method has increased tremendously in popularity. Merilä et al. (2001) reviewed 27 Q_{ST} vs. F_{ST} comparisons; only 7 years later, Leinonen, O'Hara, Cano and Merilä (2008) reviewed a total of 77 Q_{ST} vs. F_{ST} comparisons. In the latter review, most studies (48%) found $Q_{ST}>F_{ST}$, which is likely to be an effect of the subjective choice to study phenotypically divergent populations in contrasting habitats (Leinonen et al. 2008). Only two out of of the 77 comparisons unexpectedly showed $Q_{ST}<F_{ST}$ with Q_{ST} close to zero (i.e. Petit, Fréville, Mignot, Colas, Riba et al. 2001; Morgan, Evans, Garland, Swallow and Carter 2005). The authors of the respective papers suggested that strong unifying selection had acted on the studied traits.

Most plant individuals have to cope with competition by neighbours. Competitive ability is therefore an important fitness trait. Previous studies indicated that the ability to withstand competition is at least partly heritable and consequently can be selected for (Miller 1995). It is likely that there are numerous genes with epistatic interactions underlying competitive ability (Cahill, Kembel and Gustafson 2005). Relatively few studies, most of them on *Arabidopsis thaliana*, showed monogenic effects on competitive ability (Bates and Lynch 2001; Pierik, Visser, De Kroon and Voesenek 2003; Alwerdt, Gibson, Ebbs, Wood 2006). Traits that affect competitive ability are, for instance, root hair formation (Bates et al. 2001), lateral root branching (Fitter, Williamson, Linkohr and Leyser 2002), stomatal density and distribution (Alwerdt et al. 2006), shade-detection mechanisms such as perception of the red to far-red ratio by the phytochrome system (Schmitt and Wulff 1993) and ethylene sensitivity (Pierik et al. 2003). Plant size, correlating with above-ground biomass, is the most important trait affecting above-ground competitive ability (Weiner 1988; Schwinning and Weiner 1998). Due to the broad range of traits shaping competitive ability, it may be assumed that competitive ability is susceptible to genetic erosion and to mutations altering aspects of the phenotype. In addition, trade-offs between competitive ability and other traits have also been recognized, for example the trade-off with susceptibility to disease (Damgaard and Jensen 2002), with tolerance to soil compaction (Nash Suding, Goldberg and Hartman 2003) and with defense (Bazzaz, Chiariello, Coley and Pitelka 1987). Natural selection probably acts strongly on competitive ability, either by selecting against weakened competitive ability or by favouring increased competitive ability. However, an increase in competitive ability can be expected to be limited by trade-offs (Bazzaz et al. 1987; Blossey

and Nötzold 1995) and is unlikely due to rarity of favourable mutations, especially when compared to the probability of mutations which lead to decreased competitive ability. Therefore, among populations under relatively similar environmental conditions, strong unifying selection retaining competitive ability is expected. It should be noted that under unifying selection, directional selection is still a possible outcome as long as all populations evolve in the same direction, but such an unlikely scenario cannot be detected by the adopted method.

In a common garden experiment measuring variation in fitness-related traits in *Scabiosa columbaria*, Pluess and Stöcklin (2004) estimated the relative ability of plants to cope with competition as one minus the proportional difference in plant size in the treatment with *Bromus erectus* compared with the plant size achieved without the competitor. Competitive ability is therefore analogous to a relative fitness measurement (Goldberg 1996). Pluess et al. (2004) found a significant positive correlation between population-level relative competitive ability and molecular diversity as measured by randomly amplified polymorphic DNA (RAPD) fragments (r=0.65, P=0.03). The relationship between above-ground biomass under competition at the population level on the one hand and molecular diversity on the other hand was also significant (r=0.69, P=0.02), as well as the correlation between above-ground biomass and competitive ability (r_s=0.63, P=0.04). Van Treuren, Bijlsma, Ouborg and Van Delden (1993) showed that plant dry biomass of *S. columbaria* from Dutch populations, either grown with or without competition, correlated strongly with fitness. Given this correlation with fitness, the findings of Pluess et al. (2004) indicate that decreased competitive ability as well as decreased above-ground biomass under competition can be seen as a reduction in fitness. And since above-ground biomass is an important fitness-related trait with a substantial genetic basis, it is likely to be under strong unifying selection acting to preserve competitive ability.

In this study, which focuses on above-ground biomass and relative growth rate (RGR) of the length of the longest leaf of *Scabiosa columbaria* while under interspecific competition, strong unifying selection is hypothesized. As for above-ground biomass, RGR is hypothesized to show unifying selection, because increases in RGR are unlikely due to trade-offs with other plant functions (Bazzaz et al. 1987; Blossey et al. 1995) and favourable mutations are probably rare; furthermore, any decrease in RGR is disadvantageous since the rapid formation of a large rosette in *Scabiosa columbaria* is an important means to successfully compete with neighbours as new and larger rosette leaves suppress the neighbouring vegetation.
In order to detect unifying selection on above-ground biomass and relative growth rate of length of the longest leaf, data from the common garden experiment described by Pluess et al. (2004) have been reinvestigated in a Q_{ST}-F_{ST} analysis. In addition to presenting the results of these analyses, we also discuss the reliability of our conclusions, thereby contributing to the debate on Q_{ST} vs. F_{ST} comparisons.

Methods

The short-lived perennial herb *Scabiosa columbaria* L. occurs on semi-dry calcareous grasslands in Europe. It is an outcrossing species and highly susceptible to inbreeding (Van Treuren et al. 1993). Pluess et al. (2004) sampled seeds from eleven populations (9 pastures, 1 fallow and 1 hay meadow) within an area of 37 km x 11 km in the Swiss Jura and performed a common garden experiment using a half-sib approach. This allowed the calculation of additive genetic variance, not confounded by dominance and maternal effects (Leinonen et al. 2008). From each population, nine seed families were grown, with eight offspring per seed family. Half of the plants received a competition treatment with *Bromus erectus* to simulate natural field conditions. In our reinvestigation, we focus on the data from the competition treatment, i.e. four offspring per seed family. Pluess et al. (2004) performed a RAPD analysis based on which the value of G_{ST} (an F_{ST} analogue) was estimated as 0.12 with the 95% confidence interval (CI) from 0.08 to 0.16.
The length of the longest leaf was measured at the day of planting and again after 30 days.

From this data relative growth rate was calculated as the difference between natural log-transformed values divided by the difference in time, henceforth denoted RGR. Note that this is not a measure of growth of a single leaf over 30 days, but rather a measure of the rate at which the rosette size increases over time as observed from the length of the largest leaf at the respective days of measurements. At the end of the common garden experiment after 210 days, above-ground biomass, henceforth called biomass, was harvested, dried at 80 °C for 48 h and weighed. See Pluess et al. (2004) for further technical details about the common garden experiment and the RAPD analysis.

We performed a nested, mixed-model analysis of variance with type I SS using the expected mean squares method to partition variation in biomass and RGR using the formula:

$$y_{ijklmn} = \mu + \alpha_i + \beta_j + \gamma_{k(j)} + \delta_l + \zeta_{m(l)} + \varepsilon_{ijklmn},$$

where y_{ijklmn} = the phenotypic value of the nth progeny of the mth family nested in the lth population observed in the kth subplot nested in the jth plot and covarying with i, its length of the longest leaf at the start of the experiment; μ = overall mean; α_i = the length of the longest leaf at the start of the experiment; β_j = the jth plot effect; $\gamma_{k(j)}$ = the kth subplot effect nested in the jth plot; δ_l = the lth population effect; $\zeta_{m(l)}$ = the mth family effect nested in the lth population; ε_{ijklmn} = the residual error representing the within-family variation. The length of the longest leaf at the first time of measurement was used as a covariable to take into account the size variation introduced at the beginning of the experiment (e.g. maternal effects and other influences on plant size). Plot and subplot refer to randomization levels within the greenhouse and were considered fixed effects. Population and seed family were considered random effects. In order to calculate variance components of all factors, the same model was performed while treating all factors as random and using REML. Above-ground biomass was square root-transformed to improve normality of the model residuals.

Narrow-sense heritabilities (h^2) for both traits were calculated for each population separately using ANOVA models with leaf length at the start of the experiment as a covariable and family as a random factor while using the expected mean squares method. Assuming that offspring within families were related as half-sibs, we used the formula $h^2 = 4\,V_{\mathrm{FAM}}/(4\,V_{\mathrm{FAM}} + V_{\varepsilon})$ (Petit et al. 2001) where V_{FAM} is the family variance component and V_{ε} the residual variance. The mean and s.e. of the eleven population-level heritability estimates were subsequently calculated. Using the variance components of the original model, the quantitative trait differentiation was calculated as $Q_{\mathrm{ST}} = V_{\mathrm{POP}}/(8\,V_{\mathrm{FAM}} + V_{\mathrm{POP}})$ (Petit et al. 2001) where V_{POP} is the population variance component. Jackknifing over populations was performed to provide the means and 95% confidence intervals of Q_{ST}. JMP version 5.0.1.2 was used for calculations of sum of squares and variance components. Derived mean squares, F-values and P-values were calculated by hand.

Results

Seed family means of biomass ranged from 0.24 g to 1.56 g, and seed family means of RGR ranged from 3.02×10^{-3} to $25.52\times^{-3}$ proportional increase day^{-1}, resulting in a minimum doubling time of the length of the longest leaf in 28 days. The two growth traits correlated positively (Pearson's correlation, r=0.22, t_{358}=4.27, P<0.001).

Biomass and RGR did not differ among populations, whereas both traits did differ significantly among seed families within populations (Table 2.1). Plots did not differ in biomass and RGR, but subplots within plots did differ significantly in both traits (Table 2.1). The covariable length of the longest leaf at the first measurement was highly significant, but removing the covariable from the analysis did not influence the results qualitatively except that subplot within plot lost its significance for biomass (data not shown).

Concerning the variance component analysis, population differences explained almost no variation in biomass and no variation in RGR, whereas differences between seed families within populations explained 14.8% and 9.45% of the variation in biomass and RGR, respectively. Unexplained variation among individuals totalled 76.7% and 76.01% for biomass and RGR, respectively (Table 2.1). Even though the variability explained among seed families was low relative to residual variance, biomass and RGR had a

considerable level of heritability: h^2=0.31 (s.e. 0.08) and h^2=0.35 (s.e. 0.07), respectively (Table 2.1). Due to very low among-population differentiation in quantitative traits, overall Q_{ST} values were close to zero for biomass and zero for RGR, respectively, and their 95% CIs did not overlap with the one of neutral genetic differentiation (CI of G_{ST}: 0.08-0.16) (Table 2.1).

Table 2.1: Results of nested, mixed-model Type I ANOVAs of above-ground biomass and RGR of length of longest leaf. V = variance components; % = percentage variance of total; h^2 = narrow-sense heritability (s.e.); Q_{ST} = quantitative trait differentiation (95% confidence intervals).

Above-ground biomass							
		df	MS	F	p	V	%
Initial length of longest leaf		1	5.449	136.29	**0.000**	0.00101	1.93
Plot		4	0.095	1.42	0.246	0.00052	1.00
Subplot within plot		40	0.067	1.67	**0.011**	0.00277	5.31
Population		10	0.082	1.22	0.292	0.00016	0.30
Seed family within population		78	0.067	1.68	**0.002**	0.00769	14.76
Residual		226	0.040			0.03998	76.71
h^2	0.31 (0.13–0.50)						
Q_{ST}	0.004 (0.001–0.006)						
RGR of length of longest leaf							
		df	MS	F	p	V	%
Initial length of longest leaf		1	0.0037615	114.72	**0.000**	6.33×10^{-7}	1.47
Plot		4	0.0000551	0.66	0.621	2.86×10^{-13}	0.00
Subplot within plot		40	0.0000829	2.53	**0.000**	5.63×10^{-6}	13.07
Population		10	0.0000362	0.74	0.686	2.00×10^{-13}	0.00
Seed family within population		78	0.0000489	1.49	**0.012**	4.08×10^{-6}	9.45
Residual		226	0.0000328			3.28×10^{-5}	76.01
h^2	0.35 (0.20–0.51)						
Q_{ST}	0.000 (0.000–0.000)						

Discussion

Narrow-sense heritability values for biomass and RGR (Table 2.1) were intermediate when compared to experiments with other species. In *Carlina vulgaris* and *Hypochaeris radicata*, narrow-sense heritabilities for above-ground biomass were 0.156 and 0.644, respectively, and narrow-sense heritability for RGR of leaf size in *Hypochaeris radicata* was 0.481 (Becker, Berg and Matthies. 2005). Cheplick and Quinn (1988) found a narrow-sense heritability of 0.42 for above-ground biomass in *Amphicarpum purshii* and estimates ranging from 0.05 to 0.52 have been reported for different ecotypes of *Panicum virgatum* (Newell and Eberhart 1961). Among six populations of *S. columbaria* within an area of about 75 km × 100 km in southern Sweden, heritabilities differed for main stem height (mean ± s.e. 0.27 ± 0.10) and maximum height (0.43 ± 0.13) (Waldmann et al. 1998). These two traits are likely to correlate with above-ground biomass, and their heritabilities are therefore comparable to our findings.

Although the considerable heritability of biomass and RGR indicate that there is potential for evolutionary change, variation in both traits was only present among seed families within populations but not among populations (Table 2.1). This indicates that this evolutionary potential has not been used for local adaptation among the studied populations.

Caution should be taken not to overinterpret the presented genetic components of biomass and RGR, since 84.95% and 90.55% of the variability in biomass and RGR, respectively, could be attributed to differences among individuals within seed families (i.e. residual variance) and to the experimental set-up (i.e. covariable, plot and subplot effects, Table 2.1). Becker et al. (2005) likewise detected high environmental variance values for above-ground biomass in *C. vulgaris* (74%) and RGR of leaf size in *H. radicata* (78%) but a lower value for above-ground biomass in *H. radicata* (48%). Higher environmental variance components are expected for complex morphological traits or for life-history

traits compared to simple morphological traits, since the former are generally the sum of many underlying morphological traits; environmental factors act on each of these morphological traits, thereby increasing the environmental variance component and consequently decreasing the additive genetic variance component, resulting in lower heritabilities compared to those of simple morphological traits (Price and Schluter 1991). Above-ground biomass and RGR of leaf length in *Scabiosa columbaria* are likely to be the result of many underlying traits, as suggested by the substantial number of quantitative trait loci found to affect such traits (El-Lithy, Clerkx, Ruys, Koornneef and Vreugdenhil 2004; Kroymann and Mitchell-Olds 2005; Lisec, Meyer, Steinfath, Redestig, Becher et al. 2008). This polygenic nature of above-ground biomass and RGR may explain the high environmental variance.

Maternal effects may be important since the covariable in the model, initial length of the longest leaf, was highly significant. In their study on morphological variation in *S. columbaria*, Ouborg, Van Treuren and Van Damme (1991) also found strong maternal effects. However, in our study the significant covariable explained only 1.93% and 1.47% of biomass and RGR variation, respectively. Although of small effect, the inclusion of the covariate in our model increased the reliability that our Q_{ST} estimates represent additive genetic variance.

The G_{ST} value was estimated at 0.12 (Pluess et al. 2004), so the Q_{ST} values of 0.004 for biomass and 0.000 for RGR (Table 2.1) lead to the conclusion that the trait values among populations are the result of strong unifying selection pressures ($Q_{ST} < G_{ST}$). Since competitive ability is strongly related with above-ground biomass, the result for above-ground biomass can be interpreted as unifying selection for competitive ability. As stated in the introduction, unifying selection in competitive ability was expected since a decrease in competitive ability was more likely to occur than an increase in competitive ability, and a decrease in competitive ability is most likely selected against. Competitive ability is largely influenced by growth (Weiner 1988; Schwinning et al. 1998), and trade-offs with other traits, such as defence to herbivores and energy storage, limit increases in competitive ability (Bazzaz et al. 1987; Blossey et al. 1995). Since the sampled populations occur in a small area (400 km^2) within a single mountain system, abiotic and biotic conditions among them are similar, exerting similar pressure on how resources are allocated to different plant functions.

Using allozymes, Waldmann et al. (1998) also found an F_{ST} of 0.12 among the six studied Swedish populations. All of their Q_{ST} values were higher than F_{ST}, even if Q_{ST} was significantly higher than F_{ST} for only two out of eight traits: flower size and maximum height. This shows that diversifying selection on morphological traits is possible within a small sampling area with potential gene flow.

Alternative explanations for $Q_{ST} < G_{ST}$

We concluded unifying selection based on $Q_{ST} < G_{ST}$, but the validity of conclusions based on Q_{ST} vs. F_{ST} comparisons is a topic of ongoing debate. The idea that all quantitative traits would differentiate at the same rate under absence of selection, thereby achieving a Q_{ST} value equal to F_{ST}, can be questioned and there is empirical evidence against it (Morgan et al. 2005). The complexity of a trait plays a role here. Leinonen et al. (2008) suggested that Q_{ST} vs. F_{ST} comparisons are best applicable to simple quantitative traits. However, only in rare cases is an individual trait determined by only one gene, such as flower colour (Mol, Grotewold and Koes 1998) or apospory (Bicknell, Borst and Koltunow 2000). Comparing morphological traits with life-history traits, the former usually involve fewer genes, whereas life-history traits are generally the result of many genes which are potentially dominant and with more possible interactions between them (López-Fanjul, Fernández and Toro 2003; Leinonen et al. 2008). Q_{ST} vs. F_{ST} comparisons assume that quantitative traits are the result of purely additive effects, although in reality dominance, epistasis, inbreeding and maternal effects undermine these assumptions (Yang, Yeh and Yanchuk 1996; Whitlock 1999; Le Corre and Kremer 2003). With increasing complexity of the trait, the biasing effects of dominance, epistasis, inbreeding and maternal effects on Q_{ST} increase as well.

As explained in the introduction, competitive ability is probably a complex trait with epistatic

interactions (Cahill et al. 2005) and correlates with above-ground biomass (Pluess et al. 2004). Biomass and RGR are complex traits too and are known to be influenced by many lower-level traits (El-Lithy et al. 2004; Kroymann et al. 2005; Lisec et al. 2008). Thus the presented Q_{ST}-F_{ST} analysis on biomass and RGR could have been influenced by dominance, epistasis and inbreeding as possible sources of error. Maternal effects are less likely to bias the analysis of the biomass data, because we accounted for maternally inherited phenotypic variation by including intitial leaf length as a covariable. Inbreeding could not be estimated using RAPD markers, but no population was monomorphic, expected heterozygosities were comparatively high, and within-population gene diversity was normal (Pluess et al. 2004). However, the relationship of molecular diversity with mean biomass and competitive ability suggests an effect of genetic erosion, either due to inbreeding or genetic drift (Pluess et al. 2004). How could dominance, epistasis and inbreeding affect Q_{ST}? The influence of dominance, epistatis, inbreeding as well as maternal effects on Q_{ST} has not yet been tested empirically, but theoretical work (Whitlock 1999; López-Fanjul et al. 2003; Goudet and Büchi 2006; Goudet and Martin 2007) shows that dominance as well as epistatic effects generally lower Q_{ST}. Goudet et al. (2006) therefore conclude that the finding of $Q_{ST}>F_{ST}$ is reliable since possible negative effects of dominance on Q_{ST} did not affect the results qualitatively. In contrast, $Q_{ST}<F_{ST}$ can be a false inference since dominance effects could have lowered Q_{ST} below F_{ST}. Inbreeding can theoretically overcome the effect of dominance (Goudet et al. 2006), but considering the low Q_{ST} values for both traits investigated in this study, inbreeding did not seem to counteract any biasing effects of dominance.

To conclude, our result of $Q_{ST}<F_{ST}$ in both traits should be interpreted with caution, since Q_{ST} could be seriously biased downwards. However, given the large differences between Q_{ST} and G_{ST}, together with substantial heritabilities of the traits under study, we conclude that our findings are not in contradiction with the hypothesis of unifying selection.

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Chapter 3

Spatial genetic structure of *Campanula thyrsoides* across the European Alps: indications for glaciation-driven allopatric subspeciation

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Spatial genetic structure of *Campanula thyrsoides* across the European Alps: indications for glaciation-driven allopatric subspeciation

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Abstract

Quaternary climate change had profound impacts on the geographical distribution and genetic structure of plant species which is hypothesized to have triggered allopatric speciation due to spatial isolation. However, evidence is scarce despite recent advances that indicate glacial history and substrate requirements as main drivers of spatial genetic structures. Here we built upon these studies to test the role of glaciations on the morphological and ecological differentiation within the calcicolous *Campanula thyrsoides* across its European Alpine distribution range. We collected samples from 51 populations (1173 plants), used five microsatellite markers, estimated diversity (N_a, H_E) and differentiation (D_{est}, G_{ST_est}, F_{ST}) and applied Bayesian clustering analysis and tessellation methods. We found support for four genetically distinct groups of populations, arranged from West to East: i) France and Western Switzerland, ii) Central and most of Eastern Switzerland, iii) parts of Eastern Switzerland and Central Austria, and iv) Southeastern Austria, Slovenia and Northeastern Italy. Among-subspecies variance was 8.4% and each subspecies was highly differentiated (*C.*thyrsoides*: $D_{est} = 0.47$; *C.*carniolica*: $D_{est} = 0.58$). Geographic structuring of within-population diversity was not related to refugia outside of previously-glaciated terrain but to subspecies: the more thermophilic *C.*carniolica* showed significantly lower levels of within-population diversity and higher numbers of private alleles. The location of the genetic break lines between these four groups of populations correspond to well-known biogeographic barriers. However, the phylogeographic pattern has elements formerly found in both calcicolous and silicicolous species and thus questions the generality of substrate-related patterns. Within *C.*thyrsoides*, population admixture upon secondary contact may have led to high genetic diversity across the distribution range. Given the geographic and genetic differences of the subspecies we conclude that differentiation between *C.*thyrsoides* and *C.*carniolica* represents a case of glaciation-driven allopatric subspeciation reinforced by missing secondary contact due to incomplete post-glacial recolonization of potential habitats.

Keywords: Bayesian clustering, D_{ST}, genetic break lines, genetic diversity, microsatellites, tessellation

Introduction

Quaternary climate change was manifested through pronounced glacial and interglacial periods and has played a major role in changing the geographical distribution and genetic structure of plant species (Comes and Kadereit, 1998; Hewitt, 2000; Willis and Niklas, 2004). In Europe, early investigations based on floristic, palaeoecological and palaeoclimatic data have proposed locations of glacial refugia, post-glacial migration trajectories or secondary contact zones (Brockmann-Jerosch and Brockmann-Jerosch, 1926; Merxmüller, 1952, 1953, 1954; Huntley and Birks, 1983; Burga, 1988; Ozenda, 1988; Bennett et al., 1991). In recent years, molecular approaches have complemented traditional methods and have shed new light on the effect of glaciation-related range contraction, fragmentation or expansion, as well as hybridization upon secondary contact and polyploidization (e.g. Gabrielsen et al., 1997; Petit et al., 1997; Comes and Kadereit, 1998; Brochmann et al., 2004; Alsos et al., 2005; Magri et al., 2006; Petit and Vendramin, 2006; Brochmann and Brysting, 2008).

A large and detailed body of work focused on the spatial distribution of intraspecific genetic diversity of alpine plant species in the European Alps and the Carpathians (e.g. Schönswetter et al., 2002; Schönswetter et al., 2005; Mraz et al., 2007; Gugerli et al., 2008, Alvarez et al., 2009; Thiel-Egenter et al., 2009a; Thiel-Egenter et al., 2009b). Out of this suite of studies of unrelated taxa the emerging picture is that similarity in the current spatial genetic clustering of populations is sometimes strongly influenced by the substrate requirements of a species (Alvarez et al., 2009). In brief, populations of silicicolous species grouped into clusters arranged along the East–West axis of the Alps, calcicolous species showed an arrangement of clusters in the North or the South, and substrate-indifferent species revealed patterns with components of both. Additionally, the locations of the genetic break zones between the clusters often corresponded to well-known biogeographic boundaries based on floristic evidence e.g. the greater Aosta valley region (N Italy, SW Switzerland, SE France) or the border between the Western and Eastern Alps (the greater border zone of Austria, Switzerland and Italy; Ozenda, 1988; Schönswetter et al., 2005; Thiel-Egenter et al., 2009). However, the evidence for silicicolous species is far more conclusive than for calcicolous species for which only few studies exist so far (*Arabis alpina*: Ehrich et al., 2007; *Biscutella laevigata*: Parisod and Christin, 2008; *Ranunculus alpestris*: Paun et al., 2008; *Saxifraga paniculata*: Reisch et al., 2008; *Hornungia alpina*: Winkler et al., 2010; *Androsace lactea*: Schneeweiss and Schönswetter, 2010). Moreover, while substrate requirements may play an important role explaining current spatial genetic patterns in the above-mentioned widespread plants, it remains unknown to whether this holds true for rare species.

While almost all aforementioned studies on alpine plant species revealed signatures of glaciation-related genetic differentiation of populations (microevolution), much less evidence is available with regard to taxon differentiation, i.e. allopatric speciation (macroevolution), either from Europe or the Alps. Geographical isolation related to Pleistocene range shifts has been described to have caused diversification of *Reseda* species in the western Mediterranean mountains (Martin-Bravo et al., 2010). By contrast, ecological factors such as precipitation, temperature and soil types, rather than geographical isolation are supposed to have dominated Quaternary formation of Mediterranean *Cistus* species (Fernandez-Mazuecos and Vargas, 2010). Also, studies on *Paeonia* species (Sang et al., 1995; 1997) and *Saxifraga* (Brochmann et al., 1996) have detected speciation due to hybridization and polyploidization upon secondary contact since the Pleistocene glaciation. For the European Alps, glaciation-related speciation is quite possible and can be deduced from the information on current distribution of taxa (e.g. Aeschimann et al., 2004; Gugerli et al., 2008) and edaphic vicariance of closely related taxa (e.g. Ellenberg, 1996) as well as from information about concordant biogeographic and genetic break lines (see above). Nevertheless, few studies exist that focus on differentiation at the level of species (e.g. apomictic *Ranunculus cassubicus* complex: Paun et al., 2006) or subspecies (*Ranunculus alpestris*: Paun et al., 2008, *Hornungia alpina*: Winkler, et al.,

2010). For example, in both *Hornungia alpina* and *Ranunculus alpestris* congruence between phylogeographic groups and subspecies was detected and subspecies differentiation was further corroborated by contrasts in the breeding system (self-incompatibility vs. autonomous selfing; Winkler, et al., 2010).

It is well known that plant traits, such as the breeding system, dispersability or longevity, considerably influence the distribution of genetic diversity within and among populations of a species (e.g. Nybom and Bartish, 2000; Nybom, 2004). At the same time, levels of genetic diversity can be informative with respect to the glacial history in terms of reconstructing migration routes or locations of putative refugia (nunatak, periphery, lowland; Comes and Kadereit, 1998; Parisod and Besnard, 2007; Holderegger and Thiel-Egenter, 2009). In this context, it is argued that populations in long-term refugial localities such as in southern Europe harbour higher levels of genetic diversity relative to their likely descendant populations due to repeated population bottlenecks at an advancing edge of a range (Hewitt, 1996, Comes and Kadereit, 1998; but see Bialozyt et al., 2006). However, a geographic structuring is not always obvious with respect to refugia, neither in terms of diversity nor in terms of rarity of alleles, but is sometimes associated to subspecies (Paun et al., 2008, Winkler et al., 2010). Additionally, as shown for Mediterranean *Pinus* species, post-glacial migration to higher elevation may have allowed the maintenance of large effective population sizes and genetic variation in cold-tolerant species, as compared to thermophilic congenerics (Soto et al., 2010).

In this study we focus on the rare but locally abundant *Campanula thyrsoides*, a basiphilous monocarpic perennial distributed across the European Alps, the Jura Mts. and the Dinarids which is currently subdivided into two morphologically, geographically and ecologically distinct subspecies without evidence of hybrid populations (Kuss et al., 2007). This species is therefore ideally suited to investigate phylogeographic patterns of a calcicolous species with potentially glaciation-related diversification into two subspecies that have different ecological requirements. For one subspecies, *Campanula thyrsoides* subsp. *thyrsoides*, initial molecular evidence exists from the Swiss Alps that two groups of populations occur with some introgression along the well-known biogeographic line Aosta Valley – Martigny – Lake Geneva (RAPDs: Kuss et al., 2008a; microsatellites: Ægisdóttir et al., 2009). In particular, we addressed the following questions. (1) Does the alpine-wide phylogeographic pattern of *C. thyrsoides* correspond to other calcicolous species showing an arrangement of clusters in the North or the South? (2) Are the two subspecies genetically distinct such that populations group into separate geographic clusters such that allopatric speciation could be inferred? (3) Do populations outside of the formerly glaciated terrain harbor higher levels of genetic diversity relative to their likely descendant populations which recolonised previously glaciated areas?

Methods

Study species

Campanula thyrsoides L. (Campanulaceae) is a monocarpic perennial bell flower native to the European Alps, the adjacent Jura Mountains (NW) and the Dinaric Arc (SE; Aeschimann et al., 2004; Kuss et al., 2007). The species is generally rare, but locally abundant, and is predominantly found on calcareous bedrock or carbonate-bearing schist, and in subalpine to alpine meadows. *Campanula thyrsoides* is a diploid, predominantly bumblebee-pollinated outcrosser, and flowers after 3-15 years (Ægisdóttir et al., 2007; Kuss et al., 2008b; Ægisdóttir et al., 2009). Model results suggest only minuscule potential for long-distance seed dispersal (Kuss et al., 2008b). Two subspecies have been recognized that differ in morphological, ecological and chorological characteristics (Podlech, 1964; Kuss et al., 2007). Of importance for the present study are four segregating features concerning distribution, habitat, morphology and phenology: *Campanula thyrsoides* subsp. *thyrsoides* (*C.*thyrsoides*) is distributed from the NE Austrian Alps across the Alpine Arc to the SW French Alps, occurring in subalpine to alpine plant communities, has a comparably short determinate inflorescence, and flowers in June and July; *C. thyrsoides* subsp. *carniolica*

(*C.*carniolica*) is found only in the SE Alps and the Dinaric Arc, occurring in colline, montane and subalpine often ruderal or azonal scree communities, has a long indeterminate inflorescence, and flowers in July and August (Aeschimann et al., 2004; Kuss et al., 2007). In a common garden setting, the two subspecies had little overlap in flowering phenology as well as fixed morphological differences which refute the hypothesis that differences observed in the field are due to plastic responses to altitude (Kuss et al., 2007; Scheepens et al., 2010; Scheepens et al., in review). Genetic diversity data from 32 populations in the Swiss Alps based on microsatellites revealed high within-population allelic diversity (Ø H_E = 0.76), and high population differentiation (G'_{ST} = 0.53; Ægisdóttir et al., 2009). Initial evidence for spatial genetic structuring associated with post-glacial migration patterns were found for these populations (Kuss et al., 2008a; Ægisdóttir et al., 2009).

Population sampling, DNA extraction, PCR, and data scoring

We sampled 23 individuals in each of 51 populations of *C. thyrsoides* across its distribution range (41 populations of *C.*thyrsoides* and 10 populations of *C.*carniolica*; Table 3.1). This sampling included the 32 populations from the Swiss Alps mentioned above (indicated as Æ in Table 3.1). DNA extraction, PCR amplification and data handling followed the protocols outlined in Ægisdóttir et al., (2009) to assure maximum comparability. In short, DNA was extracted from 10 mg of silica-gel dried and milled (Retsch MM300; Retsch, Haan, Germany) leaf tissue using a DNeasy Plant Mini Kit (Qiagen, Hombrechtikon, Switzerland). Samples were screened using five polymorphic microsatellites (Campthy 1, Campthy 3, Campthy 5, Campthy 13, Campthy 15; Ægisdóttir et al., 2007b). Polymerase chain reaction (PCR) amplification was performed in a 10 μL reaction volume containing 15 ng of genomic DNA, 0.125 μM each of forward and reverse primers, 1 μL of 10× PCR buffer, 150 μM dNTP and 0.5 U HotstarTaq (Qiagen, Hombrechtikon, Switzerland). After a denaturation step at 95 °C for 15 min, PCR was performed for 30 cycles: 30 s annealing at a locus-specific temperature (56 °C for Campthy 1, 3, 5; 60 °C for Campthy 13 and 15), 30 s at 72 °C, and 30 s at 95 °C. The PCR ended with a final 10-min extension step at 72 °C. Horizontal gel electrophoresis of PCR products was performed using Spreadex® gels with a resolution of 2 bp in a SEA-2000TM submerged gel electrophoresis system (Elchrom Scientific AG, Cham, Switzerland). Ethidium bromide-stained (1 mg/mL) banding patterns were observed under UV light and analysed by careful manual verification of each gel at least twice. Alleles were coded according to basepair length in three digits.

All ambiguous genotyping results were repeated to minimize genotyping errors, and low quality or unreliable DNA samples and markers discarded. The genotyping error rate was calculated by re-extracting and re-amplifying 39 randomly chosen plants from the 32 Swiss populations (5.3% of the subset). Subsequently allelic differences were calculated between those 39 plants and the original datasets. The error rate was estimated to be 6.1% and was due to genotyping and scoring errors. Eventually, some individuals had to be excluded from the original dataset. The excluded individuals had microsatellite profiles with only one scored allele at a specific locus. Such loci cannot be included in analyses that require information of two alleles per locus. The reason for excluding such individuals was due to stuttering or smearing of bands (e.g. the 'second' allele could not be scored correctly) or due to evidence of null alleles (i.e. false positive homozygous genotypes). For the total dataset, the percentage of excluded data for each locus was as follows: Campthy1, 8.7% of all individuals; Campthy3, 3.2%; Campthy5, 5.1%; Campthy13, 8.4%; Campthy15, 8.5%. Nevertheless, individual profiles with only one allele can still be incorporated in population genetic programs, e.g. for estimation of population differentiation (see below).

Table 3.1: Sampling locations, geographic coordinates (WGS 84) and altitudes (metres) of 51 populations of *Campanula thyrsoides*. Taxon – thyrs.: *C. thyrsoides* subsp. *thyrsoides*, carn.: *C. thyrsoides* subsp. *carniolica*, Æ – population IDs as used in Ægisdóttir et al., (2009). Abbreviations of Swiss cantons: GR – Graubünden.

ID	Location	Taxon	Northing	Easting	Altitude	Æ
1	Switzerland: Vaud, Pres de Four	thyrs.	46°28'47"	6°06'56"	1430	4
2	Switzerland: Vaud, Col du Marchairuz	thyrs.	46°33'10"	6°15'03"	1440	1
3	Switzerland: Vaud, Les Amburnez	thyrs.	46°32'26"	6°13'56"	1340	2
4	Switzerland: Vaud, Pre du Rolle	thyrs.	46°32'45"	6°15'07"	1377	3
5	Switzerland: Vaud, Col du Jamon I	thyrs.	46°27'21"	6°58'51"	1630	5
6	Switzerland: Vaud, Col du Jamon II	thyrs.	46°27'18"	6°58'40"	1670	6
7	Switzerland: Valais, Trient, Les Tseppes	thyrs.	46°02'46"	6°58'41"	2020	8
8	France: Savoie, Col d'Iseran	thyrs.	45°25'00"	7°01'50"	2757	-
9	France: Savoie/Haut.-Alpes, Col du Galibier	thyrs.	45°03'13"	6°24'10"	2415	-
10	France: Hautes-Alpes, Col du Lautaret	thyrs.	45°02'02"	6°23'58"	2017	-
11	France: Hautes-Alpes, Le Chazelet	thyrs.	45°02'54"	6°23'58"	1766	-
12	Switzerland: Valais, Lac du Fully	thyrs.	46°10'11"	7°06'07"	2100	7
13	Switzerland: Valais, Lac du Moiry	thyrs.	46°08'17"	7°34'02"	2266	9
14	Switzerland: Bern, Stockhorn	thyrs.	46°41'28"	7°32'23"	1980	10
15	Switzerland: Bern, Schynige Platte I	thyrs.	46°39'32"	7°54'43"	1990	11
16	Switzerland: Bern, Schynige Platte II	thyrs.	46°39'16"	7°55'00"	1890	12
17	Switzerland: Uri/Valais, Furka Pass	thyrs.	46°34'35"	8°24'54"	2430	13
18	Switzerland: Uri, Unterschächen	thyrs.	46°52'55"	8°47'00"	1900	14
19	Switzerland: GR, Vals, Peil	thyrs.	46°34'53"	9°12'18"	1850	18
20	Switzerland: GR, Safiental	thyrs.	46°42'16"	9°18'24"	1857	19
21	Switzerland: GR, Medels, Parjurs	thyrs.	46°33'19"	9°18'03"	1870	20
22	Switzerland: GR, Churwalden, Joch	thyrs.	46°47'50"	9°33'53"	1890	25
23	Switzerland: GR, Langwies, Listboden	thyrs.	46°51'04"	9°45'23"	2000	15
24	Switzerland: GR, Langwies, Strassberg	thyrs.	46°50'34"	9°44'41"	1870	16
25	Switzerland: GR, Langwies, Holzbüel	thyrs.	46°49'41"	9°43'58"	1700	17
26	Switzerland: GR, St Antönien	thyrs.	46°56'38"	9°49'56"	1943	26
27	Switzerland: GR, Parsennmeder I	thyrs.	46°50'56"	9°51'07"	1995	23
28	Switzerland: GR, Parsennmeder II	thyrs.	46°50'58"	9°51'28"	1910	24
29	Switzerland: GR, Monstein, Mäschenboden	thyrs.	46°41'25"	9°47'16"	1961	21
30	Switzerland: GR, Monstein, Fanexmeder	thyrs.	46°42'03"	9°48'08"	2220	22
31	Switzerland: GR, Albula Pass, Naz	thyrs.	46°35'32"	9°45'51"	1755	28
32	Switzerland: GR, Alp Laret	thyrs.	46°30'41"	9°50'22"	2180	27
33	Switzerland: GR, Schuol, La Motta	thyrs.	46°48'42"	10°16'28"	2142	29
34	Switzerland: GR, Ftan, Prui	thyrs.	46°48'25"	10°13'24"	2100	30
35	Switzerland: GR, Tschlin, Alp Tea I	thyrs.	46°53'46"	10°26'04"	2200	31
36	Switzerland: GR, Tschlin, Alp Tea II	thyrs.	46°53'38"	10°25'43"	2150	32
37	Austria: Tyrol, Rüfikopf	thyrs.	47°12'00"	10°10'08"	2307	-
38	Austria: Tyrol, Jöchelspitze	thyrs.	47°16'47"	10°22'10"	2226	-
39	Austria: Tyrol, Elmer Kreuzspitze	thyrs.	47°20'41"	10°34'34"	1874	-
40	Austria: Tyrol, Rinnen	thyrs.	47°24'23"	10°42'46"	1215	-
41	Austria: Tyrol, Hintertux	thyrs.	47°06'46"	10°39'05"	2011	-
42	Austria/Italy: Carinthia/Udine, Plöckenpass	carn.	46°36'02"	12°57'05"	1629	-
43	Italy: Udine, Sella Nevea	carn.	46°23'35"	13°27'46"	932	-
44	Slovenia: Upper Carniola, Nemski Rovt	carn.	46°16'23"	13°58'30"	663	-
45	Slovenia: Upper Carniola, Pleasa	carn.	47°17'55"	13°58'54"	950	-
46	Slovenia: Inner Carniola, Postojna	carn.	45°49'37"	14°14'25"	515	-
47	Austria/Slovenia: Carinthia, Loibelpass	carn.	46°25'51"	14°15'38"	1068	-
48	Austria: Carinthia, Zell-Freibach	carn.	46°29'15"	14°26'42"	833	-
49	Slovenia: Lower Styria, Sklendrovec	carn.	47°06'22"	14°59'56"	339	-
50	Slovenia: Lower Styria, Vitanje	carn.	46°22'27"	15°17'16"	422	-
51	Slovenia: Lower Styria, Brodnice	carn.	46°06'24"	15°16'53"	283	-

Data analysis

We used a Bayesian clustering method implemented in Structure 2.3.3 (Pritchard et al., 2000; Falush et al., 2003; Pritchard and Wen, 2004) to test the hypothesis that subsets of populations form discrete groups (Structure

parameters: admixture model, no prior population information, independent allele frequencies in each population, burnin = 106, MCMC = 106, log-likelihood (Ln P) of K =1-6 clusters of populations, 20 independent replicates). We followed the protocol by Evanno et al. (2005) to estimate the most probable number of K groups of populations based on $\Delta(K)$, i.e. the mean absolute difference of the second order rate of change with respect to K. In each of the 100 runs, individuals were assigned to one of the K groups which then translated into group membership probabilities for each population, i.e. proportional assignment. Following the protocol by Thiel-Egenter et al., (2009) we then calculated pairwise Euclidean distance matrices for every K = 2–6 and every replicate, i.e. 100 matrices, using R 2.10.0 (R Development Core Team, 2010). These distance matrices were imported into the software Barrier (Manni et al., 2004) which allows visualising the geographic location of genetic breaks between populations using Monmonier's maximum-difference algorithm (Monmonier, 1973). The robustness of the genetic breaks was then quantified by bootstrapping the 100 matrices obtained above. The spatial genetic structure of all 51 populations according to the most probable number of K clusters was mapped using ArcGIS 9.0 (ESRI, Redlands, CA, USA) with thematic shape files on maximum glacial ice extent by Schönswetter et al. (2005). To contrast the results obtained from the combined Structure/Barrier analysis we carried out spatial analysis of molecular variance (samova; Dupanloup et al., 2002). However, the latter method failed to detect any geographic pattern and only separated single disconnected peripheral populations of *C. thyrsoides* with increasing K (data not shown and not further discussed).

All further statistics were performed in R (R Development Core Team, 2010). There is an ongoing debate which measure of genetic differentiation is more appropriate to understand causes or consequences of population structure especially when data is based on loci with high mutation rates such as microsatellites (Whitlock, 2011). We therefore anaylzed genetic differentiation within clusters of populations using several metrics and compare these. We calculated the bias-corrected Dest (Jost, 2008; Gerlach et al., 2010) and bias-corrected G_{ST_est} (Nei and Chesser, 1983; Gerlach et al., 2010) with 1000 bootstraps each using the R library 'demetics'. We refrained from computing standardized G_{ST}, also known as G'_{ST} (sensu Hedrick, 2005) because G'_{ST} approaches D_{est} when diversity is high (Jost, 2008), as in our case. We also calculated F_{ST} using the R library 'ade4' (Thioulouse et al., 1997).

The distribution of molecular variance within and among populations and putative K clusters (amova; Excoffier et al., 1992) was also calculated using 'ade4'. Sample-size corrected measures of within-population diversity (N_a: mean number of alleles; H_E: expected heterozygosity) were calculated based on multiple random reductions (Leberg, 2002). Differences in within-population diversities among groups of populations were assessed using anovas and Tukey's honest significant difference tests.

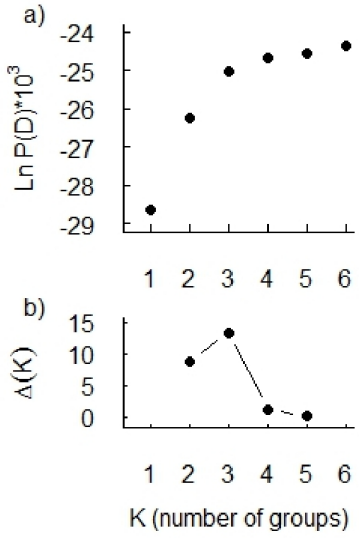

Figure 3.1: Estimating the most probable number of groups of population (K) for *Campanula thyrsoides* based on Bayesian clustering for K =1–6 number of groups and 20 runs each (Structure: Pritchard et al., 2000). a) Mean log-likelihood of the data (Ln $P(D)$) per K, i.e. standard output from Structure. Standard deviations are too small to show at this scale. b) Mean absolute difference of the second order rate of change with respect to K, i.e. $\Delta(K)$ (following Evanno et al., 2005).

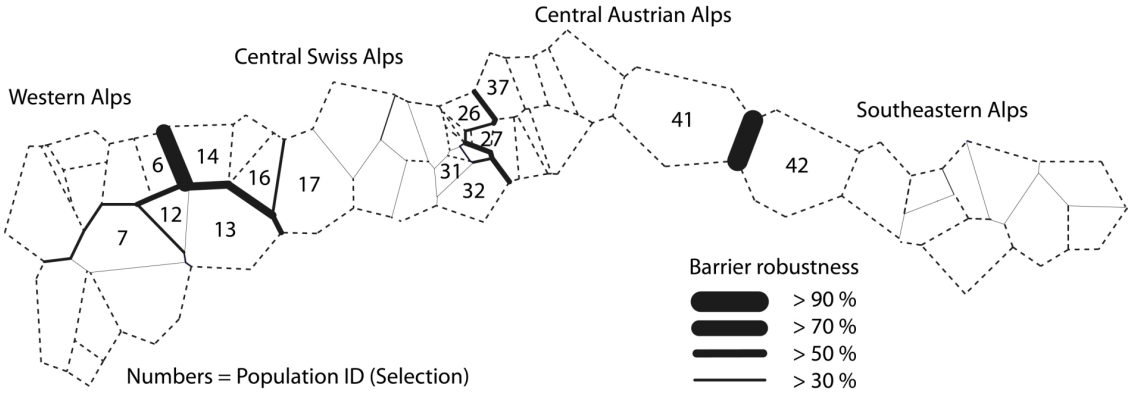

Figure 3.2: Results of geographical identification of intraspecific genetic breaks in *Campanula thyrsoides* in the European Alps and adjacent Jura Mts. (BARRIER: Manni et al., 2004). The thickness of break segments represents their value of robustness, ranging from 1% to 100%. Dotted lines are the outlines of the Voronoï tessellation polygons in which centres the population are located (see Fig. 3.3, Table 3.1).

Results

Spatial genetic pattern

Bayesian cluster analysis gave a step-by-step optimization of the log-likelihood of the data (Ln $P(D)$) with an approximate saturation plateau at K =3 or K =4, i.e. three groups of populations are clearly defined, one additional group is less distinct (Fig. 3.1a). Based on Evanno's ad hoc estimator $\Delta(K)$, three groups of populations are present in our data (Fig. 3.1b). However, the spatially explicit mapping of the K =2-6 groups revealed three dominant genetic barriers separating *C. thyrsoides* in four larger geographic groups (Fig. 3.2). The strength of the genetic barrier between groups increased with increasing K (not shown). A clear separation of the Southeastern populations from the remaining populations was supported in 90% of the cases (based on 100 distance matrices). This split represents also the separation between the two subspecies, i.e. *C.*carniolica* in the Southeastern Alps and *C.*thyrsoides* in the rest of the sampled area. The second genetic break is located in a zone including parts of Western Switzerland, the Southern Jura Mts. and Northeastern Savoie (France). Here, values of robustness range between 40-83% with one dominant break line and a number of trailing ones. The third split among populations is found in Eastern Switzerland with barrier robustness ranging from 28-31%. Also in this case, one dominant break line was found with a number of subordinate ones. No other split was convincingly supported.

Based on these results we plotted group membership probabilities for each population based on K =4 and the run with the highest posterior probability (out of 20 runs; Fig. 3.3). The four groups of populations are clearly visible. Strong discontinuities of colours between populations indicate strong genetic barriers between these populations and vice versa (see Fig. 3.2). The least supported break line in Eastern Switzerland represents a zone of gradual introgression of alleles from populations East and West of this line (Fig. 3.3).

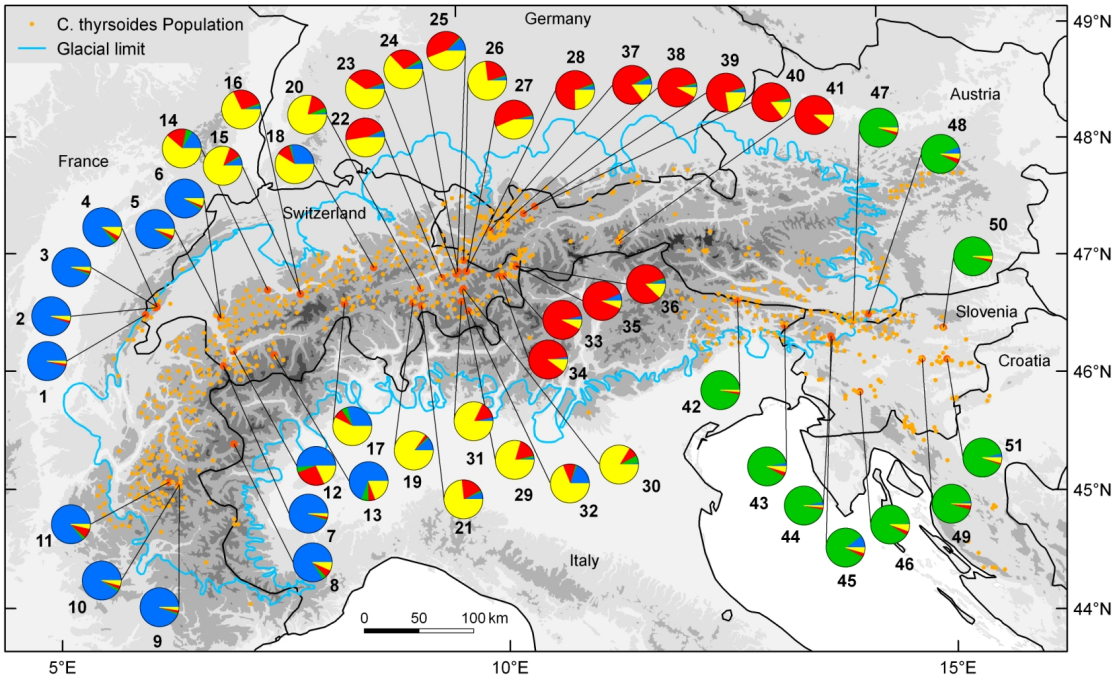

Figure 3.3: Spatial genetic structure of *Campanula thyrsoides* in the European Alps and adjacent Jura Mts. based on probabilities of Bayesian population clustering using K=4 and the run (out of 20) with the highest posterior probability (see Fig. 3.1). Numbers indicate population IDs. Map projection: Mollweide; geographic coordinates: WGS 84.

Genetic diversity

Overall genetic differentiation of *C. thyrsoides* populations was high with D_{est} = 0.58 (Table 3.2). Looking at the subspecies, populations of *C.*carniolica* were also highly differentiated, equal in magnitude to the overall differentiation (D_{est} = 0.58). Populations of *C.*thyrsoides* were well differentiated (D_{est} = 0.47) but less pronounced than *C.*carniolica*. In all cases, the genetic differentiation among populations within different groups was significant. The values of $G_{\text{ST\_est}}$ and F_{ST} were identical. Overall, $G_{\text{ST\_est}}$ and F_{ST} values were 3.4× to 4.0× lower than the respective values for D_{est} though they mirror the findings for D_{est} in terms of order of differentiation and significance.

Molecular variation in the genetic data as attributed to within-population variation was ranging from 79.4 to 89.7% (Table 3.3). Between 8.0 and 12.2% of the variation was found among populations of the differently sized groups. The variation among groups of populations was highest when testing the contrast between the two subspecies, i.e. 8.4%. This was even higher than the among group variation for the entire data set, i.e. 6.7%. For the three groups of *C.*thyrsoides* populations, little variation was detected between the two central regions, i.e. 2.3%.

The number of alleles within each of the four groups of populations was very similar and the number of private alleles was low, though slightly higher in *C.*carniolica* populations (Western Alps: n_{tot}=67, n_{private}=2; Central Swiss Alps: n_{tot}=69, n_{private}=1; Central Austrian Alps: n_{tot}=64, n_{private}=1; Southeastern Alps: n_{tot}=65, n_{private}=4). In terms of standardised allelic richness, we found no difference among the three groups of *C.*thyrsoides* populations (df=2, SS=2.35, F=2.06, p>0.05; Table 3.4). However, *C.*carniolica* populations were significantly less diverse than *C.*thyrsoides* (df=1, SS=4.61, F=7.82, p<0.01), which

was due to a significant difference between *C.*carniolica* populations and *C.*thyrsoides* from the Central Swiss Alps ($p<0.01$). Within a subspecies all analysed populations originated from either within or outside of previously glaciated terrain, respectively. Therefore, we found no differences in genetic diversity between populations sampled outside of the previously glaciated terrain, i.e. *C.*carniolica*, and those from recolonised areas (*C.*thyrsoides*) when corrected for subspecies. Additionally, within subspecies, no obvious geographic structure of diversity was found that could be attributed to potential refugia (Fig. 3.4). Essentially the same results were found for differences in heterozygosity (H_{E},) and are thus omitted here. As a side note, genetic diversity within populations (N_{a}, H_{E}) was not found to be related to altitude, neither across nor within subspecies, nor within groups of *C.*thyrsoides* (linear regression; all $p>0.05$).

Table 3.2: Measures of genetic differentiation of different groups of *Campanula thyrsoides* populations. Given are mean values and 95% confidence intervals based on 1000 bootstraps. '**' denotes significance value $p<0.001$. WA: Western Alps (pops 1-13); CSA: Central Swiss Alps (pops 14-26, 29-32); CAA: Central Austrian Alps (pops 27, 28, 33-41); SEA: Southeastern Alps (pops 42-51).

	D_{est}	$G_{\mathrm{ST\_est}}$ (=F_{ST})
All populations	0.58±0.014**	0.15±0.001**
*C.*carniolica* (SEA)	0.58±0.03**	0.17±0.004**
*C.*thyrsoides* (WA, CSA, CAA)	0.47±0.013**	0.12±0.023**
*C.*thyrsoides* (CSA, CAA)	0.33±0.015**	0.09±0.002**
*C.*thyrsoides* (WA)	0.43±0.025**	0.11±0.004**
*C.*thyrsoides* (CSA)	0.32±0.02**	0.08±0.004**
*C.*thyrsoides* (CAA)	0.27±0.022**	0.07±0.003**

Discussion

Phylogeographic pattern

The results from the cluster analysis suggest that the species *C. thyrsoides* consists of at least three genetically defined groups ($\Delta(K)$; Fig. 3.1b). A fourth cluster is potentially present based on visual interpretation of the log-likelihood of the data (Fig. 3.1a). Spatial mapping of the genetic break lines indicate the existence of four rather than three clusters (Fig. 3.2) because a gradient of introgression seems to be present in Eastern Switzerland which coincides spatially with the well-known biogeographic barrier between the Eastern and Western Alps (see below). The introgression likely blurred the difference between groups of populations East and West of this break line (Figs. 3.2 and 3.3). We therefore have reasons to assume that four groups of populations are present in our data.

The West to East arrangement of the four clusters corresponds well to the pattern seen in many other alpine species (e.g. Schönswetter et al., 2005; Alvarez et al., 2009). Nevertheless, the observed North to South orientation of the three genetic break lines has been found mostly in silicicolous species such as *Geum reptans* (Thiel-Egenter et al., 2009; Frei et al., in review) or *Phyteuma betonicifolium* (Schönswetter et al., 2002). The notable exception is the silicicolous congeneric *Campanula alpina* for which the arrangement of clusters is from North to South (Ronikier et al., 2008). In our study species, the calcicolous *Campanula thyrsoides*, the arrangement of clusters is from West to East, and therefore does not corroborate a general arrangement of clusters from North to South as detected in other calcicolous species, e.g. *Arabis alpina* (Ehrich et al., 2007) or *Hornungia alpina* (Winkler et al, 2010). In our study we were not able to include *C.*thyrsoides* populations from the NE Alps which would potentially support a large Central/Northern Austrian Alpine group or a fifth cluster to the North of *C. *carniolica* (Fig. 3.3). In either case, the arrangement of clusters would then follow both West to East, as well as North to South and therefore have elements of a 'silicicolous' and a 'calcicolous' pattern (Alvarez et al., 2009). The alternative grouping of the NE populations together with the SE *C.*carniolica* as shown for vicariance in *Androsace lactea* (Schneeweiss and Schönswetter, 2010) seems unlikely given the geology-induced distribution gap and the strong genetic differentiation found between *C.*carniolica* in

the Southeast and *C.*thyrsoides* from the Central Austrian Alps. The phylogeographic pattern found for *C. thyrsoides* is so far unique for a calcicolous species and questions the generality of a 'silicicolous' and a 'calcicolous' pattern as outlined by Alvarez et al. (2009).

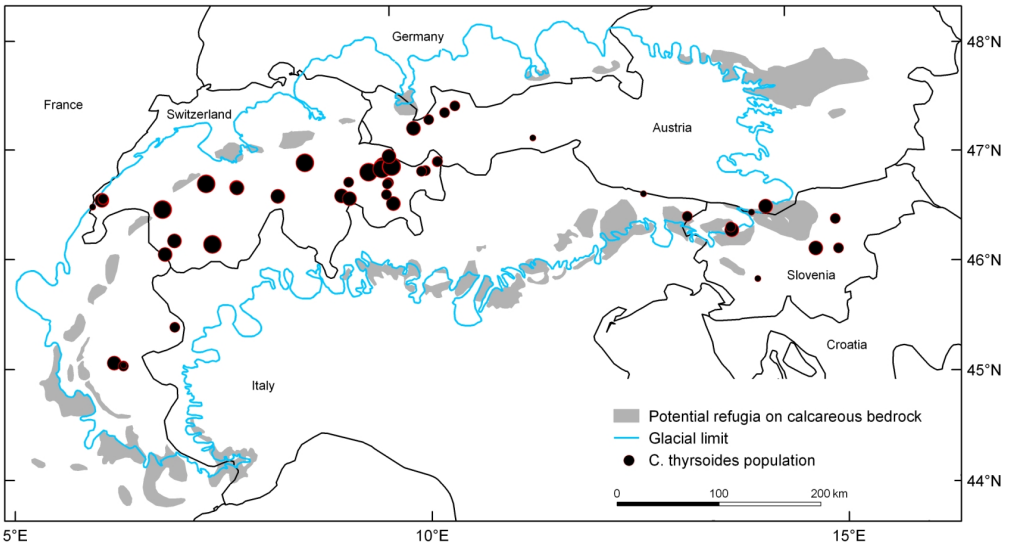

Figure 3.4: Patterns of within-population genetic diversity (N_a) in 51 populations of *Campanula thyrsoides* from the Alps and the Jura Mts. The size of the dots is directly proportional to the amount of genetic diversity (see Table 3.4). Grey areas indicate potential glacial refugia on calcareous bedrock (adapted from Schönswetter et al., 2005). Map projection: Mollweide; geographic coordinates: WGS 84.

Table 3.3: Analysis of Molecular Variance (AMOVA) for $K = 4$ clusters of *Campanula thyrsoides* populations and the hierarchy of barrier robustness (see Figs. 1 and 2). df: degrees of freedom; SS: sum of squares; Var. (%): Explained variability (%); SEA: Southeastern Alps (pops 42-51); WA: Western Alps (pops 1-13); CSA: Central Swiss Alps (pops 14-26, 29-32); CAA: Central Austrian Alps (pops 27, 28, 33-41). *C.*car.* = *C.*carniolica*; *C.*thy.* = *C.*thyrsoides.*

	df	SS	Var. (%)
All			
Among groups	3	214.9	6.7
Among populations	47	848.0	10.1
Within populations	1645	3089.8	83.2
SEA (*C.*car.*) vs. rest (*C.*thy.*)			
Among groups	1	99.9	8.4
Among populations	563	848.0	12.2
Within populations	1645	3090	79.4
WA vs. CSA/CAA			
Among groups	1	85.1	6.0
Among populations	39	359.9	10.5
Within populations	1387	2642.9	83.5
CSA vs. CAA			
Among groups	1	29.9	2.3
Among populations	26	205.8	8.0
Within populations	952	1820.7	89.7

Table 3.4: Within-population genetic diversity and cluster assignment probability for 51 studied population of *Campanula thyrsoides* across five polymorphic microsatellite loci. ID: population ID; n: number of samples without missing alleles; N_a: standardised mean number of alleles per population (based on 100 multiple random reductions; Leberg, 2002); H_E: expected heterozygosity; $P(.)$: assignment probability to each of the four clusters based on the run (out of 20) with the highest posterior probability (WA: Western Alps; CSA: Central Swiss Alps; CAA: Central Austrian Alps; SEA: Southeastern Alps; see Fig. 3.3).

ID	n	N_a	H_E	P(WA)	P(CSA)	P(CAA)	P(SEA)
1	21	4.4	0.679	0.95	0.02	0.02	0.01
2	13	5.4	0.775	0.86	0.08	0.04	0.03
3	23	5.8	0.785	0.94	0.03	0.02	0.01
4	22	5,6	0.766	0.94	0.03	0.02	0.01
5	20	6.9	0.832	0.91	0.06	0.02	0.02
6	20	6.6	0.810	0.90	0.05	0.03	0.02
7	15	6.1	0.761	0.94	0.03	0.02	0.01
8	8	5.6	0.719	0.84	0.06	0.06	0.03
9	14	4.7	0.611	0.94	0.03	0.02	0.01
10	16	5.4	0.661	0.91	0.04	0.03	0.02
11	17	6.3	0.743	0.86	0.04	0.08	0.03
12	17	5.9	0.783	0.51	0.18	0.26	0.05
13	18	7.0	0.851	0.69	0.19	0.06	0.06
14	16	7.2	0.867	0.17	0.61	0.17	0.05
15	19	5.8	0.796	0.04	0.68	0.26	0.02
16	19	5.7	0.746	0.07	0.81	0.11	0.01
17	13	6.3	0.819	0.30	0.57	0.08	0.05
18	20	7.3	0.853	0.28	0.59	0.11	0.02
19	16	6.1	0.783	0.11	0.85	0.03	0.01
20	19	4.8	0.721	0.02	0.78	0.15	0.04
21	19	6.1	0.780	0.07	0.73	0.19	0.01
22	17	6.6	0.808	0.06	0.47	0.46	0.01
23	20	6.6	0.779	0.06	0.63	0.29	0.02
24	17	6.7	0.813	0.12	0.44	0.43	0.01
25	18	6.6	0.773	0.05	0.60	0.35	0.01
26	20	5.7	0.755	0.04	0.73	0.22	0.01
27	19	6.6	0.804	0.04	0.25	0.70	0.02
28	19	6.4	0.805	0.03	0.44	0.52	0.01
29	17	5.0	0.718	0.02	0.80	0.17	0.02
30	18	5.3	0.760	0.02	0.84	0.08	0.07
31	21	5.0	0.732	0.04	0.81	0.14	0.01
32	21	6.0	0.766	0.19	0.69	0.11	0.01
33	18	5.6	0.767	0.04	0.07	0.88	0.01
34	20	5.4	0.731	0.03	0.10	0.87	0.01
35	17	5.3	0.779	0.04	0.12	0.82	0.02
36	16	5.5	0.769	0.07	0.06	0.86	0.01
37	12	5.9	0.740	0.06	0.15	0.77	0.02
38	16	5.0	0.711	0.03	0.08	0.88	0.02
39	9	5.2	0.763	0.04	0.22	0.72	0.01
40	16	5.3	0.741	0.02	0.14	0.82	0.02
41	16	3.8	0.547	0.01	0.11	0.88	0.01
42	17	4.6	0.682	0.01	0.01	0.02	0.96
43	12	4.8	0.719	0.04	0.04	0.04	0.89
44	12	5.8	0.712	0.03	0.01	0.02	0.94
45	8	5.0	0.694	0.10	0.04	0.03	0.82
46	19	4.0	0.612	0.01	0.06	0.03	0.90
47	12	3.9	0.709	0.02	0.04	0.03	0.92
48	12	6.0	0.812	0.06	0.04	0.04	0.86
49	15	5.8	0.725	0.02	0.01	0.03	0.93
50	16	5.3	0.717	0.01	0.02	0.02	0.94
51	10	5.3	0.711	0.04	0.04	0.01	0.91

The strength of the genetic break lines suggest different levels of gene flow between adjacent groups of populations which may translate into different degrees of spatial isolation. Most pronounced is the genetic break between the Central Austrian Alpine group and the Southeastern Alpine group (Fig. 3.2; Tabs. 3.2 and 3.3) which coincides with the largest distribution gap (Fig. 3.3) within the species. A pronounced genetic break is also found between the continously distributed populations of *C.*thyrsoides* from the Western Alpine and Central Swiss Alpine groups (Fig. 3.2, Tab. 3.3) which is not associated with morphological differences (Scheepens et al., in review). It is therefore likely that spatial isolation led to allopatric subspeciation within *C. thyrsoides*. The timing of this process remains obscure without a molecular clock and there is so far no evidence that the differentiation of *C.*carniolica* and *C.*thyrsoides* preceded the genetic differentiation within *C.*thyrsoides* as shown for *Androsace lactea* (Schneeweiss and Schönswetter, 2010).

We found that only a small portion of the molecular variation of *C. thyrsoides* based on microsatellite markers was associated with the split into four groups of populations (6.7%) or the split into two subspecies (8.4%; Table 3.3). Our results show considerably lower values than what has been reported for many other alpine plant species with corresponding phylogeographic clustering which were investigated using AFLP markers, e.g., 51.2% in *Phyteuma globulariifolium* (Schönswetter et al., 2002), 40.7% in *Ranunculus glacialis* (Schönswetter et al., 2004), 39.8% in *Veronica alpina* (Albach et al., 2006), or 29% in the *Ranunculus alpestris* group (Paun et al., 2008). However, in a study comparing both types of markers applied to the same set of samples, AFLP markers revealed more phylogeographic structuring than microsatellites (Skrede et al., 2009). Possible reasons may be the much higher number of AFLP loci usually surveyed and/or high mutation rates in microsatellites leading to homoplasy. As described earlier, in our case, the four groups of *Campanula thyrsoides* populations were considerably well supported by Bayesian clustering (Fig. 3.1) and had pronounced genetic barriers between them (Fig. 3.2).

Also, in *C. thyrsoides*, 8-12.2% of the variation was attributed to variation among populations within groups, leaving 80-90% of the variation within populations (Table 3.3). This suggests at least moderate levels of gene flow among populations over considerable distances. Results from our previous experimental work support high levels of within-population gene flow due to an almost exclusively outcrossing breeding system and locally restricted pollinator movement, i.e. bumblebees (Ægisdóttir et al., 2007; Ægisdóttir et al., 2009). At the same time previous work also showed low levels of modeled long-distance seed dispersal (Kuss et al., 2007). It is noteworthy that restricted gene-flow was found in a fine-scale genetic analysis of 24 mostly small populations in a 10km^2 area of the Swiss Alps (Frei et al., in revision). Here, small populations separated by less than 50m were considerably differentiated, i.e. $G'_{\mathrm{ST}} = 0.32$. Thus, the experimental and fine-scale genetic data mentioned above suggest restricted gene flow among populations even at close proximity which contradicts somewhat the at least moderate levels of gene-flow found in the range-wide data set. Nevertheless, direct observation of pollen flow and modelled results for seed dispersal very likely underestimate gene flow, while genetic differentiation may exist even with low levels of gene flow.

In our data set, genetic differentiation among populations was pronounced, both within groups (D_{ST} = 0.27-0.58) and across groups (D_{ST}= 0.58). By contrast, measures of $G_{\mathrm{ST\_est}}$ and F_{ST} for the same groups of populations were 3.4× to 4× lower than D_{ST} (Table 3.2). The observed mismatch between D_{ST} and G_{ST} is in line with recently published theoretical considerations and practical simulations (Jost, 2008; Gerlach et al., 2010). However, while the latter authors deduce from their work that interpretations on genetic differentiation based on the commonly used indices G_{ST} and F_{ST} need to be reconsidered, we found that the hierarchical order of differentiation was identical between D_{ST} and $G_{\mathrm{ST\_est}}$ /F_{ST} and showed comparable levels of significance (see also Whitlock, 2011). Therefore, the conclusions based on either index would

remain the same.

Glacial refugia and post-glacial migration

The current distribution of *C.*thyrsoides* is almost exclusive in the Northern Alps, i.e. North of the Central Alpine divide or West of it in the south-western Alps, while *C.*carniolica* is restricted to the Southeast (Fig. 3.3; Kuss et al., 2007). From this pattern we would deduce that the main locations of glacial refugia were along the Northern rim of the Alps and the Southeast, respectively. Figure 3.3 also indicates that populations of *C.*carniolica* in the Southeastern Alps are mostly found in locations that were outside or at the periphery of the previously glaciated area (blue line; van Husen, 1987; Voges, 1995). By contrast, the great majority of *C.*thyrsoides* populations are found within the area of the last glacial maximum ice extent from which we infer pronounced post-glacial migration. In situ survival during the glaciation(s) seems therefore unlikely for *C.*thyrsoides* and more probable for *C.*carniolica*. Nevertheless, our study remains inconclusive with respect to the origin of *C.*thyrsoides* populations because the differentiation among the three phylogroups is not too strong. Therefore, extant populations of *C.*thyrsoides* could be derived either from glacial refugia North of the Alps, from the Western Alps/Jura, and/or Southwestern Alps, and/or from the Northeastern refugium.

Our initial hypothesis was that genetic diversity would be higher in populations at or in close proximity to their glacial refugium. However, we did not find an obvious geographic aggregation of populations of *C.*thyrsoides* with higher genetic diversity along or towards the putative peripheral Northern refugia (Fig. 3.4). One reason could be that gene flow between divergent lineages from separate refugia upon secondary contact led to high heterozygosity and genetic diversity (Petit et al., 2003). Based on Bayesian cluster analysis we could detect high levels of gene flow across the biogeographic barrier in Eastern Switzerland (CSA vs. CAA) and to a lesser degree in Western Switzerland (WA vs. CSA; Fig. 3.3). It is likely that similar levels of gene flow are also present within a cluster of populations, e.g. Central Swiss Alps, as indicated by the low genetic differentation within a cluster (Tab. 3).

Additionally, current gene flow and demography can be much more decisive for genetic diversity within populations than historical events (e.g. Amos and Harwood, 1998; Nybom et al., 2004). For the outcrossing *C. thyrsoides* with a pronounced generation overlap, we would rule out population size as a dominant variable explaining variability in genetic diversity since we found no significant relationship across several magnitudes of population sizes (Kuss et al., 2008a; Kuss et al., 2008b; Ægisdóttir et al., 2009). Obviously, the structure in our data is strongly influenced by the two subspecies such that within-population diversities need to be discussed in the light of the different habitat ecology and potentially different glacial histories. *C.*carniolica* is a colline-montane-subalpine while *C.*thyrsoides* a subalpine-alpine subspecies (Kuss et al., 2007) and the current ranges may be the result of range contraction and range expansion, respectively. As shown for Mediterranean *Pinus* species, post-glacial migration to higher altitudes and into vast expanses of previously glaciated terrain may have allowed the maintenance of large effective population sizes and genetic variation in cold-tolerant species, as compared to thermophilic congenerics (Soto et al., 2010). A similar situation could explain the lower genetic diversity found in *C.*carniolica* populations. Nevertheless, differences in genetic diversities between the subspecies should not be overstressed since significant contrasts were only found between two out of four groups of populations, i.e. *C.*thyrsoides* from CSA and *C.*carniolica* from SEA.

Conclusions

The spatial genetic pattern of the calcicolous *C. thyrsoides* suggests at least four geographically distinct genetically defined groups with little or no overlap and a clear separation of the two subspecies. The locations of genetic break lines between these groups of populations correspond to well-known biogeographic barriers. The genetic signature of postglacial recolonization of *C. thyrsoides* in the Alps and Jura Mts. is not blurred by current and past

seed and pollen flow. However, the phylogeographic pattern has elements of both calcicolous and silicicolous species and thus questions the generality of substrate-related patterns. Given the geographic and genetic differences of the subspecies we conclude that differentiation between *C.*thyrsoides* and *C.*carniolica* represents a case of glaciation-driven allopatric subspeciation during the last glacial maximum reinforced by missing secondary contact due to incomplete post-glacial recolonization of potential habitats.

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Chapter 4

Glacial history and adaptation explain regional differentiation in phenotypic traits in a widespread Alpine plant

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Glacial history and adaptation explain regional differentiation in phenotypic traits in a widespread Alpine plant

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Abstract

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 the monocarpic plant *Campanula thyrsoides*. A common garden experiment with 21 populations from across the Alps and Jura Mts. revealed differentiation among four phylogeographic regions in morphology, phenology and response to clipping. Regional differences remained after accounting for effects of isolation by distance and altitude of origin on phenotypic traits, indicating that glacial history is at least partly responsible for regional phenotypic differentiation. We further asked whether this differentiation is due to adaptation. Delayed flowering in plants from the Southeastern Alps indicated adaptation to long submediterranean summers, and contrasts with early-flowering plants experiencing short growing seasons in the high Alps to the west. A clipping treatment indicated low susceptibility to grazing in plants from the Western Alps, which is in accordance with their predominant occurrence in pastures and meadows. Alpine-wide adaptation to local environmental conditions is suggested by correlations between number of inflorescences, inflorescence height and biomass with altitude of origin. Finally, Q_{ST}-G'_{ST} analyses suggested past unifying selection for all traits except biomass.

Keywords: *Campanula thyrsoides*, common garden, genetic drift, phenology, Q_{ST}-F_{ST} analysis

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. 2010). Numerous molecular studies demonstrated that glacial history left a genetic signature in 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; Parisod 2008; Paun et al. 2008; Alvarez et al. 2009; Thiel-Egenter et al. 2009). It can be hypothesised that widespread Alpine plants are affected by glacial history not only in differentiation of neutral molecular markers, but in a similar pattern also in differentiation of

phenotypic traits through processes of selection and drift (Hewitt 1996). Although studies comparing molecular and phenotypic differentiation are nowadays common, in particular to investigate past selection pressures (e.g. Galen et al. 1991; Petit et al. 2001; Leinonen et al. 2008; Banta et al. 2007; Ramírez-Valiente et al. 2009), in this study we explicitly investigate whether glacial history, which includes drift and selection, affected phenotypic differentiation in a widespread Alpine plant.

Phenotypic differentiation among populations of a species can be caused by stochastic processes or selection (Lande 1976). Stochastic processes include mutations, neutral genetic drift through small population size, bottlenecks and founder effects, and gene flow via dispersal of seeds and pollen (Nei et al. 1975). Selection is non-random as it favours survival and reproduction of certain phenotypes over others (Fisher 1930). It is likely that both random and selective processes have affected phenotypic traits of widespread plants from the European Alps, for several reasons: 1) the time scale of glacial cycles is large enough for genetic drift and mutations to arise (Hartl and Clark 1995; Klekowski 1997; Schönswetter et al. 2005); 2) founder effects are likely during migration out of and into the Alps (Gugerli et al. 2001; Hewitt 2004); 3) refugia, 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; 4) the independent evolutionary history of populations in isolated refugia caused neutral differentiation which may have allowed for subsequent differential adaptation during and after recolonisation of the different regions of the Alps. We hypothesise that glacial history led to phenotypic differentiation among phylogenetic lineages still visible today, and we test this hypothesis using a common garden experiment. Since, besides stochastic processes, selection is hypothesised to be a potential constituent of effects of glacial history, we perform a Q_{ST}-F_{ST} analysis to test for past selection processes (Spitze 1993; Merilä and Crnokrak 2001) and we perform trait-altitude correlations to investigate current adaptation to altitude.

Common garden experiments allow the quantification of genetic trait differentiation among regions, populations and seed families (Clausen et al. 1948) and are therefore an appropriate method to investigate effects of glacial history on phenotypic differentiation. By introducing a treatment to the experiment, the plasticity of traits with respect to the treatment can be determined (Fischer et al. 2000; Pluess and Stöcklin 2004). We performed a common garden experiment with 21 populations of the monocarpic *Campanula thyrsoides* from across the European Alps. *C. thyrsoides* is genetically subdivided into four major phylogeographic regions located longitudinally across the European Alps and Jura Mts. (Kuss et al. 2007; Ægisdottir et al. 2009; Kuss et al. 2011) as has been found in other species (e.g. Schönswetter et al. 2005; Alvarez et al. 2009). This is congruent with major biogeographic distribution patterns based on floristic data (Merxmüller 1952, 1953, 1954; Ozenda 1988). The phylogeographic regions found with molecular markers are roughly located (i) in the 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; Kuss et al. 2011).

As stated above, we hypothesise that differentiation in phenotypic traits should be observed among phylogeographic regions as a result of glacial history, whether this be through drift or past selection processes. Although we remain largely neutral with regard to the directions of differentiation due to selection, we additionally hypothesise that particularly strong differentiation will be observed between the Southeastern Alps and the other regions. Molecular studies suggested that the Southeastern lineage may have survived *in situ* under environmental conditions contrasting with those in the other regions where populations probably survived *ex situ* (Kuss et al. 2011). This may have 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. Since flowering phenology

strongly changes with altitude, we propose that adaptation to the respective climates has most likely occurred in phenological traits (Rathcke and Lacey 1985; Körner 2003).

Methods

Study species

Campanula thyrsoides L. (Campanulaceae) is a monocarpic bell flower distributed across the European Alps, including the lower mountain ranges of the Dinarids, and in the Jura Mts. (Aeschimann et al. 2005). The species occurs 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. unpublished) 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 taller subsp. *carniolica* in the Dinarids (Podlech 1964; Scheepens et al. 2011; in SEA). The species' altitudinal distribution typically ranges from 1600 to 2200m a.s.l. (Kuss et al. 2007), but reaches lower altitudes in the Jura Mts. and Dinarids, with the lowest recorded population at 217m a.s.l. near Gracnica, Slovenia (Jürg Stöcklin, personal observation). The species is characterised by isolated populations containing several hundred to a few thousand individuals (Kuss et al. 2008). Initiation of flowering is rosette-size dependent, and Kuss et al. (2008) estimated the average flowering age at about 10 years using integral projection models as well as herb chronology. However, the 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 is mostly self-incompatible (Ægisdóttir et al. 2007).

Common garden experiment

Six seed families each from 21 populations were sampled across four phylogeographic regions in the Alps and the Jura (WA, CSA, CAA, SEA; Fig. 4.1, Appendix Table 4.5). These phylogeographic regions were chosen based on the genetic structure revealed by Bayesian clustering analysis using 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 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 snail grazing.

On 19 May 2008, plants were transplanted to a common garden located in Davos, Graubünden, Switzerland (N 46°47'06.97", E 9°48'57.02") at 1530m a.s.l. The site, formerly used as an organically fertilised subalpine meadow-pasture, was ploughed before the plants were transplanted into the local soil. Out of 1008 plants, a total of 953 plants could be transplanted (Appendix Table 4.5) as there were missing individuals in several populations due to mortality in the greenhouse. The plants were distributed among four blocks each with (ideally) two members of each seed family. Rain-

fall at the common garden location is 1026 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 with electric wire with mesh size 15 x 15 cm and the 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.

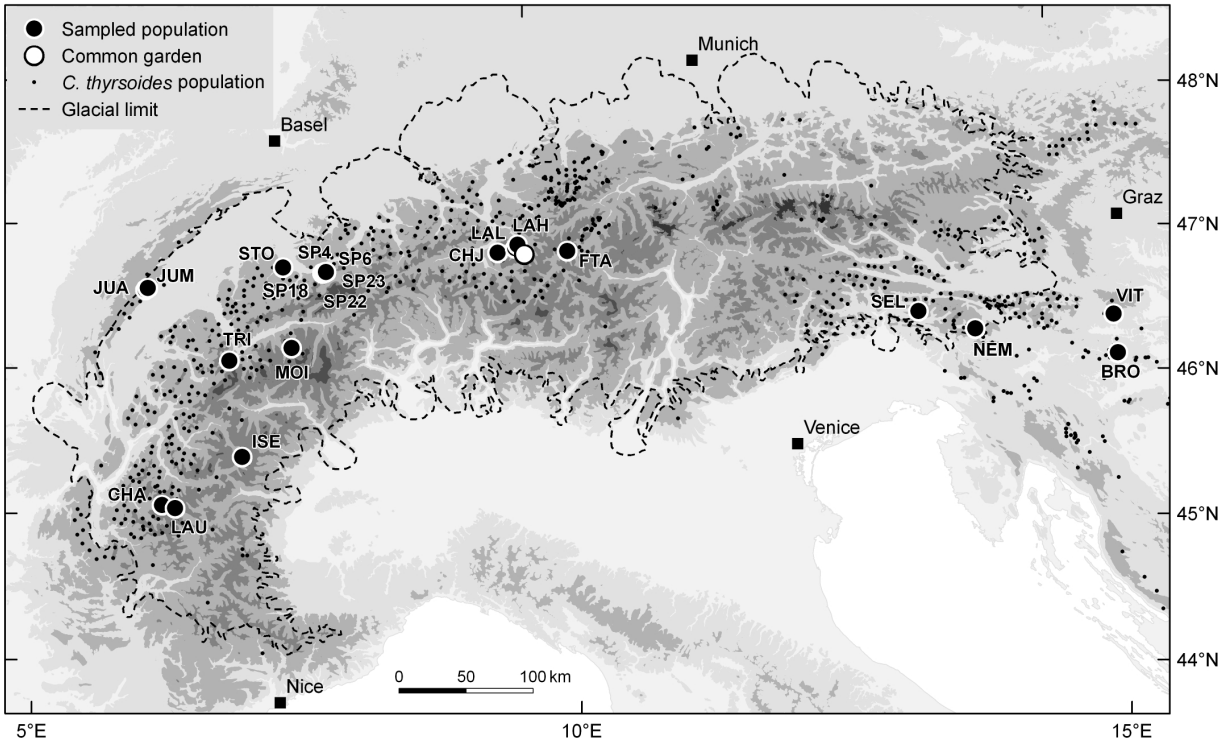

Figure 4.1: Map showing the 21 sampled populations, the common garden location, the distribution of *Campanula thyrsoides* and the glacial limit from the last glacial maximum. JUA, JUM, CHA, LAU, ISE, TRI and MOI belong to phylogeographic lineage WA; STO, SP4, SP6, SP18, SP22 and SP23 belong to CSA; CHJ, LAL, LAH and FTA belong to CAA; SEL, NEM, VIT and BRO belong to SEA.

Generalised linear mixed 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-grond biomass, were analysed using generalised linear mixed models (GLMMs; Crawley 2007). We applied Type I sums of squares, which has the properties that, firstly, the predicted sums of squares of the whole model are completely decomposed, irrespective of whether the model is balanced (i.e. orthogonal) or not. Secondly, it allows to remove certain effects before others, which is preferable in unbalanced nested designs. The drawback is that the order matters 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 differences in initial size affecting 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 excluded from all models. The clipping treatment was included as a fixed effect in all models, except for 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; Bolker et al. 2009). 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 deletion of the interactions and climbing up 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) (Bates 2005; Crawley 2007). Tukey's HSD tests were used to locate differences between region pairs.

Any observed regional differentiation could be an effect of (i) alpine-wide distance-related effects of drift or of adaptation to gradually changing environmental conditions or of (ii) adaptation to local environmental conditions, particularly climatic conditions related with altitude. To test whether regional differentiation remained when these confounding factors were removed, we included either (i) the distance from sampled population to the common garden or (ii) the altitude of the population origin as covariate in the models, positioned directly after rosette diameter. 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).

We treated the number of flowers and above-ground biomass as potentially adaptive traits as well as proxies for fitness, so we assume that

any differentiation in these fitness-related traits is due to life-history divergence among the regions through drift or selection as opposed to, for instance, inbreeding in the populations of origin.

Response to clipping

We included a clipping treatment to simulate grazing or mowing because *Campanula thyrsoides* occurs in grazed and mown grasslands and may be affected to different degrees by these regimes. 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 (*sensu* Snaydon 1991; Pluess and Stöcklin 2005). This value was subtracted from unity to obtain a measure of the relative ability to withstand clipping, where lower values indicate a stronger susceptibilty. 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 mean trait values of unclipped plants, calculated from seed family means, and altitude of population origin (Appendix Table 4.5), which was used as a proxy for environmental variables related with altitude, probably mostly climate. To investigate the effect of the two subspecies on the correlation, we tested a subset without SEA populations, thus containing only subsp. *thyrsoides*. In order to test if any correlations remained when the effect of phylogeographic region was removed, we performed correlations between altitude of origin and residuals of population mean trait values obtained by ANOVA using region as explanatory variable. Significancy of multiple correlations was estimated using a sequential Holm-Bonferroni correction (Holm 1979).

To investigate the presence of additive genetic variance in the phenotypic traits, narrow-sense heritabilities (h^2) were calculated for each population with unclipped plants only, using $h^2 = V_{\mathrm{FAM}}/(4\,V_{\mathrm{FAM}} + V_{\mathrm{e}})$. V_{FAM} was the variance component at the seed family level and V_{e} was the residual variance (Petit et al. 2001), obtained from simple models including rosette diameter and seed family as random variables. The use of half-sibs precluded dominance and genetic maternal effects to influence the results (Leinonen et al. 2008). To investigate whether regional differentiation in traits is the result of natural selection, differentiation in traits among all populations was quantified as Q_{ST} (Spitze 1993; Merilä and Crnokrak 2001) using $Q_{\mathrm{ST}} = V_{\mathrm{POP}}/(8\,V_{\mathrm{FAM}} + V_{\mathrm{POP}})$ (Petit et al. 2001) where V_{POP} was the population variance component obtained from simple models including rosette diameter, population and seed family as random variables. Q_{ST} was then compared with the neutral molecular differentiation indices $G_{\mathrm{ST\_est}}$ (Nei 1983), $G'_{\mathrm{ST\_est}}$ (Hedrick 2005) and D_{est} (Jost 2008). Q_{ST} values of various traits were calculated for unclipped plants only, following Petit et al. (2001), and 95% confidence intervals of Q_{ST} values were obtained by using Jackknife bootstrapping over populations (O'Hara and Merilä 2005). The neutral genetic differentiation indices $G_{\mathrm{ST\_est}}$, $G'_{\mathrm{ST\_est}}$ and D_{est} were calculated for the sampled populations using previously obtained microsatellite data (Ægisdóttir et al. 2009; Kuss et al. 2011) in SMOGD 1.2.5 (Crawford 2010) with 1000 bootstrap replications to determine their 95% confidence intervals. Instead of the Schynige Platte populations SP4, SP6, SP18, SP22 and SP23, which were not genotyped, we used genetic data from two other populations nearby as a replacement (SPO and SPU in Ægisdóttir et al. 2009; population no. 15 and no. 16 in Kuss et al. 2011).

Except for neutral genetic differentiation indices, 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 and Maechler 2009).

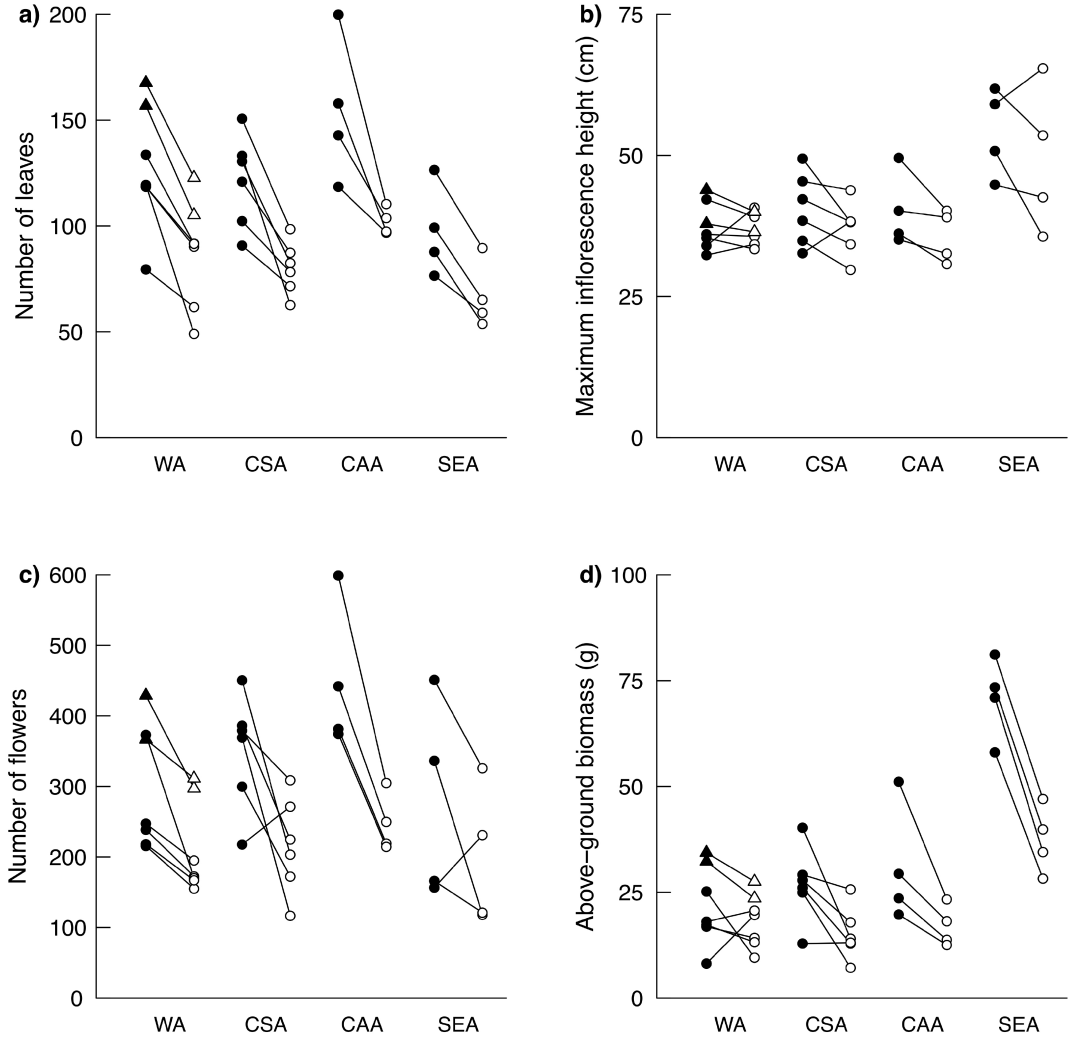

Figure 4.2: Reaction norms of populations to the clipping treatment on *Campanula thyrsoides* plants in the common garden ordered by phylogeographic lineage for (a) number of leaves, (b) maximum inflorescence height, (c) number of flowers, and (d) above-ground biomass. Filled circles indicate the control plants, open circles indicate the clipped plants. Triangles indicate the two populations from the Jura Mts. *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

Results

Phenotypic differentiation

Significant regional differentiation was present in all traits (Table 4.1, Fig. 4.2) and the variance explained by phylogeographic region ranged from 0% (leaf length to width ratio 2009) to 24% (maximum inflorescence height). Post-transplantation survival was higher in CSA compared to WA, number of leaves was higher in CAA than in SEA, maximum inflorescence height was higher in SEA than in WA, and SEA populations had higher above-ground biomass compared to the other regions (Table 4.2, Fig. 4.2). 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 4.2), 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.

Clipping significantly reduced all trait values except for leaf length to width ratio in 2008 (Tables 4.2 and 4.3). A clipping×region in-

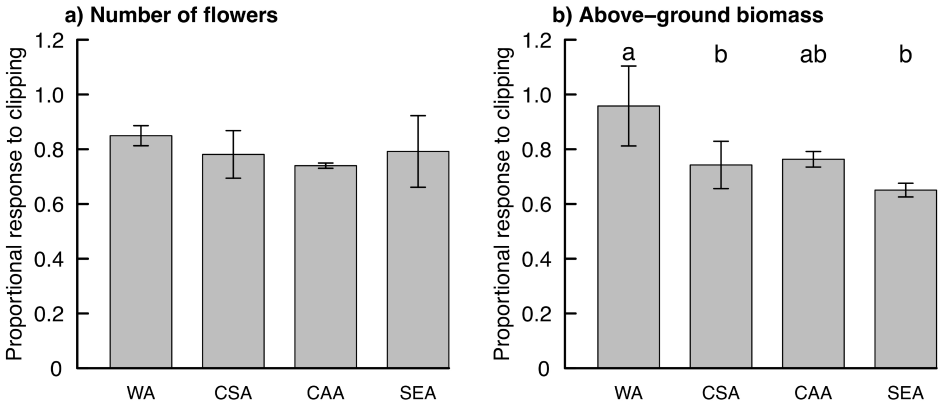

Figure 4.3: Mean proportional response to clipping per phylogeographic lineage of *Campanula thyrosides* plants in the common garden for (a) number of flowers, and (b) above-ground biomass. Means ± 1 SE are shown. Letters denote significant differences at α=0.05 obtained with a Tukey's HSD test. *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

teraction 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 4.1). Several populations responded positively to clipping in maximum inflorescence height, number of flowers and above-ground biomass (Fig. 4.2). When measured as the proportional seed family reduction due to clipping, the response in maximum inflorescence height (data not shown) and number of flowers (Fig. 4.3a) did not differ among regions, but biomass of plants from CSA and SEA was significantly reduced by clipping compared to WA (Fig. 4.3b).

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 4.1). Noteworthy is that of WA populations, the two populations from the Jura generally had higher values for number of leaves and above-ground biomass (Fig. 4.2). The Jura populations also reached numbers of inflorescences comparable with SEA, 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 amount of variation (Table 4.1). There was a significant clipping×population interaction for leaf length to width ratio in 2009 and maximum inflorescence height (Table 4.1), 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 it was highly variable (Table 4.1). 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) had only negligable influence on the results as long as interactions did not precede main factors contained in these interactions (results not shown).

Table 4.1: Results of 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. See Methods section for details on GLMMs. Chi^2 values and their significancies were obtained from model comparisons. *%VC*—Variance components were obtained from analyses with all factors treated as random effects. df—degrees of freedom, residual df varies per trait due to mortality and due to flowering traits being recorded in flowering plants only.

	Post-transplantation survival				Number of leaves			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	479.0	***	64.2	1	156.7	***	16.3
Clipping treatment	1	-		-	1	144.7	***	23.5
Region	3	20.1	***	17.2	3	69.0	***	2.6
Population (Region)	1	2.3		4.1	1	71.0	***	7.3
Seed family (Population)	1	1.0		0.6	1	4.2	*	2.2
Clipping×Region	3	-		-	3	4.4		0.0
Clipping×Pop (Region)	1	-		-	1	0.1		0.4
Clipping×Seed family (Pop)	1	-		-	1	0.5		2.8
Residuals	942			13.9	789			44.9
	Leaf length to width ratio 2008				Leaf length to width ratio 2009			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	94.3	***	4.0	1	52.1	***	3.3
Clipping treatment	1	1.1		0.2	1	36.6	***	6.4
Region	3	49.7	***	9.0	3	9.6	*	0.0
Population (Region)	1	108.8	***	17.7	1	55.2	***	7.5
Seed family (Population)	1	19.5	***	8.4	1	9.6	**	7.9
Clipping×Region	3	2.4		0.0	3	2.2		0.0
Clipping×Pop (Region)	1	0.1		0.8	1	4.3	*	5.5
Clipping×Seed family (Pop)	1	0.0		0.0	1	0.0		0.0
Residuals	790			60.0	755			69.4
	Number of inflorescences				Maximum inflorescence height			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	15.4	***	0.1	1	52.5	***	0.0
Clipping treatment	1	54.4	***	1.6	1	20.9	***	2.7
Region	3	34.9	***	1.0	3	128.9	***	23.9
Population (Region)	1	70.7	***	3.5	1	81.6	***	13.0
Seed family (Population)	1	15.6	***	2.3	1	19.2	***	9.7
Clipping×Region	3	2.4		0.0	3	2.2		0.0
Clipping×Pop (Region)	1	1.8		0.0	1	7.8	*	0.0
Clipping×Seed family (Pop)	1	0.0		0.0	1	6.7	**	5.3
Residuals	672			91.5	672			45.4
	Number of flowers				Above-ground biomass			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	34.9	***	0.3	1	85.1	***	10.4
Clipping treatment	1	93.9	***	21.5	1	72.8	***	14.8
Region	3	24.8	***	0.8	3	90.0	***	15.9
Population (Region)	1	35.0	***	9.9	1	14.4	***	3.8
Seed family (Population)	1	0.0		1.6	1	4.8	*	4.7
Clipping×Region	3	7.6	(*)	1.0	3	18.7	***	3.0
Clipping×Pop (Region)	1	0.0		1.9	1	1.1		1.8
Clipping×Seed family (Pop)	1	0.0		0.0	1	0.0		0.0
Residuals	609			62.9	681			45.5

(*) $P = 0.054$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4.2: Mean values of morphological traits of *Campanula thyrsoides* per phylogeographic region and treatment (control versus clipped plants) in the common garden. Means (SE) of regions are based on population means, which in turn are based on seed family means. Different letters indicate significant differences ($\alpha = 0.05$) among regions using Tukey's HSD tests and between treatments based on significancy of the treatment factor in the model (Table 4.1). *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

	Region			
	WA	CSA	CAA	SEA
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))
Leaf length to width ratio 2009	7.25 (0.42)	6.86 (0.36)	7.26 (0.45)	7.18 (0.57)
Number of leaves (%)	106.3^{ab} (9.7)	100.0^{ab} (6.7)	128.3^{a} (9.9)	82.4^{b} (9.1))
Number of inflorescences	4.06 (0.55)	4.86 (0.23)	4.65 (0.31)	6.22 (0.87))
Maximum inflorescence height (cm)	37.21^{a} (1.26)	38.86^{ab} (2.21)	37.58^{ab} (2.5)	52.33^{b} (4.62)
Number of flowers	254.7 (31.0)	285.5 (23.3)	335.3 (33.7)	241.1 (66.4)
Above-ground biomass (g)	18.89^{a} (2.67)	20.96^{a} (2.62)	23.15^{a} (4.52)	53.40^{b} (4.44)
	Treatment			
	Control	Clipped		
Post-transplantation survival (%)	-	-		
Leaf length to width ratio 2008	2.92 (0.15)	2.99 (0.16)		
Leaf length to width ratio 2009	7.70^{a} (0.10)	6.77^{b} (0.24)		
Number of leaves (%)	126.0^{a} (11.7)	84.1^{b} (7.4)		
Number of inflorescences	5.6^{a} (0.4)	4.3^{b} (0.5)		
Maximum inflorescence height (cm)	43.1^{a} (3.8)	39.8^{b} (3.2)		
Number of flowers	347.8^{a} (38.3)	217.9^{b} (10.3)		
Above-ground biomass (g)	38.1^{a} (11.1)	21.9^{b} (5.2)		

Phenology

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. 4.4). At the second measurement of 2009, the majority of surviving plants from WA, CSA and CAA were ripening, whereas only 7% of SEA plants reached the ripening stage and 83% were flowering (Fig. 4.4). Most SEA plants had finished flowering only on 20 October 2009, when snow and frost hampered further growth.

Out of the total 953 plants, 132 plants 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 4.2 and 4.3). 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.

By including either the distance of sampled populations to the common garden or altitude as covariate in the models, we excluded supposedly recent effects of drift and adaptation in populations across the European Alps. By doing so, highly significant regional differentiation was retained for all traits (Appendix Table 4.6 and 4.7), indicating that regions showed phenotypic differences which relate to historic effects of drift and adaptation caused by glacial history. Other factors were generally not influenced qualitatively except for population, which lost significance in models with the altitude covariate and with number of inflorescences, number of flowers and above-ground biomass as response variable due to the removal of among-population differences by the covariate (Appendix Table 4.7).

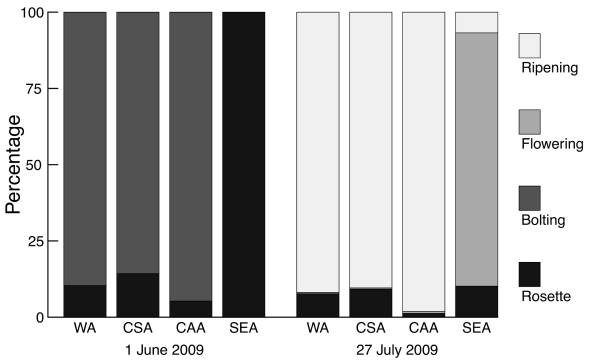

Figure 4.4: Percentage of plants in four distinct phenological stages (rosette, bolting, flowering, ripening) of surviving *Campanula thyrsoides* plants in the common garden from four phylogeographic regions at two census dates. *WA* Western Alps, *CSA* Central Swiss Alps, *CAA* Central Austrian Alps, *SEA* Southeastern Alps.

Local and regional 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 4.3). When SEA populations were omitted, the direction of correlations did not change and the correlation coefficients remained substantial albeit non-significant. When the region effect was removed, correlations generally became stronger (Table 4.3).

Narrow-sense heritability h^2 was substantial for all traits, ranging from 0.069–0.440 (Table 4.4a), although about half of the examined populations completely lacked heritability in specific traits. Q_{ST} for seven phenotypic traits ranged from 0.127-0.664 (Table 4.4a), whereas Hedrick's G'_{ST_est} was 0.596 (95% CI 0.559-0.634; Table 4.4b). For all investigated traits except above-ground biomass, 95% confidence intervals of Q_{ST} did not overlap the confidence interval for G'_{ST_est}, indicating unifying selection on phenotypic traits among all populations, whereas no selection pressure need to be invoked to explain the differentiation observed in above-ground biomass. Jost's proposed measure of genetic differentiation D_{est} was estimated at 0.537 (95% CI 0.502-0.572; Table 4.4b) and is therefore comparable with G'_{ST_est}, although it would marginally overlap with leaf length to width ratio in 2009 and with number of flowers. Nei's index of neutral differentiation, G_{ST_est}, yielded a much lower value, 0.127 (95% CI 0.110-0.144; Table 4.4b). For all traits except number of leaves and inflorescences, this would result in lower marker differentiation than quantitative trait differentiation, and therefore would lead to the opposing conclusion that these traits are under diverging selection.

Table 4.3: Correlations between measured plant traits in the common garden and altitude of origin in *Campanula thyrsoides*. Correlations involved (i) all populations, (ii) without SEA, and (iii) without SEA on the residuals of a model testing the effect of phylogeographic lineage on plant traits. r—Pearson's correlation coefficient.

	All populations		Without SEA		Without SEA and region	
	r	P-value	r	P-value	r	P-value
Post-transplantation survival	0.13	0.56	-0.26	0.32	-0.40	0.11
Leaf length to width ratio 2008	-0.38	0.088	-0.24	0.36	-0.28	0.27
Leaf length to width ratio 2009	-0.27	0.23	-0.53	0.027	-0.54	0.027
Number of leaves	0.26	0.26	-0.46	0.061	-0.50	0.040
Number of inflorescences	-0.70	0.00039 *	-0.64	0.0052	-0.76	0.00036 *
Maximum inflorescence height	-0.82	5.1×10^{-6} **	-0.46	0.064	-0.53	0.027
Number of flowers	0.04	0.85	-0.50	0.042	-0.67	0.0030
Above-ground biomass	-0.93	10×10^{-9} ***	-0.61	0.010	-0.70	0.0017 *

Sequential Holm-Bonferroni corrected P-values comparable to: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$

Table 4.4: Heritability and comparisons between phenotypic and molecular differentiation for several traits in *Campanula thyrsoides* as measured in a common garden experiment. a) Phenotypic differentiation, h^2—narrow-sense heritability averaged over all populations; Q_{ST}—quantitative genetic differentiation (95% CI) among all populations; ↓—unifying selection when compared to G'_{ST_est}; ns—non-significant difference with G'_{ST_est}. b) Molecular differentiation. Mean (95% CI) of G_{ST_est}, G'_{ST_est} and D_{est} among all 21 populations based on microsatellite data.

a) Phenotypic differentiation	h^2	Overall Q_{ST}
Leaf length to width ratio 2008	0.315	0.268 (0.254-0.282) ↓
Leaf length to width ratio 2009	0.337	0.439 (0.365-0.513) ↓
Number of leaves	0.440	0.199 (0.115-0.284) ↓
Number of inflorescences	0.069	0.127 (0.116-0.137) ↓
Maximum inflorescence height	0.231	0.395 (0.382-0.409) ↓
Number of flowers	0.359	0.388 (0.255-0.522) ↓
Above-ground biomass	0.210	0.664 (0.591-0.738) ns
a) Molecular differentiation		
G_{ST_est}		0.127 (0.110-0.144)
G'_{ST_est}		0.596 (0.559-0.634)
D_{est}		0.537 (0.502-0.572)

Discussion

Phenotypic differentiation

The detected regional differentiation in vegetative and reproductive traits (Tables 4.2 and 4.3, Fig. 4.2) as well as in the response to clipping (Fig. 4.3) and in flowering phenology (Fig. 4.4) in *Campanula thyrsoides* is in line with the observed phylogeographic structure based on microsatellite data which previously confirmed four longitudinally oriented regions (Kuss et al. 2011). These results therefore support our hypothesis that glacial history caused regional differentiation in several phenotypic traits across the Alps in this species. Geographic differentiation in phenotypic traits has been found frequently since the pioneering study by Clausen, Keck and Hiesey (1948), but has usually been related to adaptation to specific environmental conditions, most notably along clines or among regions differing in climate (e.g. Prock and Körner 1996; Joshi et al. 2001; Del Pozo et al. 2002; Olsson and Ågren 2002; Santamaría et al. 2003; Ramírez-Valiente et al. 2009). In contrast, studies suggesting a role for genetic drift in phenotypic trait differentiation are less common (for a review see Leinonen et al. 2007), whether due to the pervasiveness of natural selection or due to a research bias to investigate phenotypically diverged populations under contrasting selection pressures (Merilä and Crnokrak 2001). Additionally, phylogeographic studies are generally restricted to neutral molecular markers and, in the context of effects of glacial history, do not compare findings to phenotypic divergence in phylogeographic lineages (Schönswetter et al. 2005; Besnard et al. 2007, but see Lagercrantz and Ryman 1990), except when including multiple species for which phenotypic differentiation has already been established by taxonomy (Paun et al. 2008; Thiel-Egenter et al. 2009).

After including either the distance from the sampled populations to the common garden or altitude of origin as a covariate in the models, region remained significant. This suggests that differentiation is present in these traits beyond any potential confounding effect of regional differentiation due to (i) relatively recent distance-related effects of drift or of adaptation to gradually changing environmental conditions across the European Alps or to (ii) adaptation to climatic conditions related to altitude. This differentiation in phylogeographic lineages, not obscured by any recent effects, is thus likely a result of historic effects of drift and adaptation caused during survival of glaciations in refugia or during recolonisation after the retreat of glaciers.

Local and regional adaptation

Differentiation in phenotypic traits can be due to genetic drift or adaptation, or a combination of both. First we checked if patterns of genetic

differentiation in phenotypic traits as observed in the common garden could reasonably be explained by adaptation (e.g. as home-site advantage). Second, in order to strengthen claims of adaptation, we also checked (1) for correlations between phenotypic traits with altitude of origin, a proxy for climate, (2) for evidence of past selection pressures as indicated by Q_{ST} vs F_{ST} comparisons.

Adaptation to regional conditions could have played a role in the differentiation of observed traits. One clear example is the delayed flowering of SEA plants (ssp. *carniolica*) (Fig. 4.4), 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 and Lacey 1985; Weber and Schmid 1998; Olsson and 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 environment (Scheepens et al. 2011). In a previous study, we found that specific leaf area in *C. thyrsoides* was also regionally differentiated, which was mainly due to SEA plants reaching significantly lower values compared to plants from the other regions, which we explained as an adaptation to the drier submediterranean climate (Scheepens et al. 2010).

An ultimate cause for the strong morphological and phenological differentiation between the subspecies *carniolica* (SEA) and *thyrsoides* (WA, CSA, CAA) could be that subsp. *carniolica* survived glaciation *in situ* in a markedly different climate at lower altitude compared to subsp. *thyrsoides*, which most likely survived in refugia along the northern rim of the Alps, causing differential adaptation to their respective climates (Jäger 2000; Kuss et al. 2007; Kuss et al. 2011).

Home-site advantage in the common garden

Home-site advantage occurs when the populations from the region in which the common garden is located (CAA) achieve higher fitness compared to populations from outside this region. This would suggest that populations from the various regions have diverged in life history what leads to maladaptive growth and lower fitness in foreign environments (Galloway and Fenster 2000; Becker et al. 2006). Our results may hint at such a home-site advantage, as populations from CAA (where Davos is located) had the highest mean reproductive output estimated as number of flowers, although not significantly different from the other regions due to high variance (Fig. 4.2, Table 4.2). This optimal performance is likely an effect of higher energy reserves at time of flowering, which is also indicated by CAA plants having the highest number of leaves in 2008 (Table 4.2). Additionally, the trend of higher post-transplantation survival in CSA and CAA compared to WA and SEA (Table 4.2) may be partly explained as a home-site advantage, since region explained 17% of variation in survival (Table 4.1). That glacial history, and not just recent effects of drift and selection, caused these patterns of differentiation is strongly suggested from the models with distance to the common garden or altitude of origin added as covariate, which retained highly significant effects of phylogeographic region.

In line with these results, the two central regions were weakly differentiated (2.3%) compared to differentiation between other groups of regions based on microsatellite markers (Kuss et al. 2011). Gene flow between the two central regions may therefore be substantial, whereas the deep Aosta Valley may restrict dispersal between WA and the other regions, and the absence of *C. thyrsoides* in most of South Tyrol (see distribution map in Kuss et al. 2007) separates the Southeastern Alpine region from those to the west. Concluding, the home-site advantage as observed for survival and reproductive output suggests regional adaptation, and weak molecular differentiation between populations from the two central regions may explain similarities in performance.

Response to clipping

Although many studies investigate 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 long-term grazed and ungrazed sites (Warwick and Briggs 1979; 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 and Aguiar 2008; Suzuki 2008).

In this study, plants from different regions responded differently to the clipping treatment (Table 4.1). WA plants were less affected by clipping compared to plants from other regions, where clipping generally had negative effects on traits (Figs. 4.1 and 4.2). Some populations even benefitted from clipping (i.e. overcompensation; McNaughton 1983). *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, personal observation), thus potentially being subject to regular grazing or mowing. Although we do not have data on grazing pressure in the investigated populations which would allow more reliable tests, the results of this study indicate differentiation in susceptibility to clipping, which may explain the respective habitat preference in this region. Recent adaptation seems a more likely explanation than glacial history, but the ultimate cause for the regional structuring could be drift during glacial survival.

Population level differentiation

Populations within regions were differentiated for all measured traits except for survival (Table 4.2). This population differentiation could be partly due to neutral genetic drift, partly to adaptation to the population-specific environment (Clausen et al. 1948; Joshi et al. 2001; Ramírez-Valiente et al. 2009) as the Alpine landscape is spatially and temporally heterogeneous (Körner 2003) and populations can have small size and can thus be subject to considerable drift (Stöcklin et al. 2009). Since variance explained by population was higher than by phylogeographic region in five out of eight traits (Table 4.1), it could be argued that population-specific conditions had more influence on recent adaptation than effects of glacial history had on phylogeographic differentiation.

The two populations from the Jura Mts., although belonging phylogenetically to the Western Alps, stood out with higher number of leaves, number of flowers, biomass (Fig. 4.2) and number of inflorescences in comparison to other WA populations (data not shown). 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. The different plant communities in which *C. thyrsoides* occurs in the Jura Mts. compared to the Alps (see methods), but also climatic differences, e.g. the longer snow-free season at this comparably lower altitude, may pose different selection pressures. Interestingly, the molecular marker-based phylogeography strongly suggests that *C. thyrsoides* populations from the Jura and the French Alps originate from the same refugium (Ægisdóttir et al. 2009; Kuss et al. 2011). Even increasing the number of potential phylogenetic groups with one or two in the spatial genetic structure analysis of Swiss populations by Ægisdóttir et al. (2009) did not break down this Western Alpine group. Therefore, the phenotypic differentiation between populations from the Jura Mts. versus those from the Alps is likely a result of divergent selection.

The clipping×population interaction for leaf length to width ratio in 2009 and maximum inflorescence height (Table 4.1) indicates that these traits responded differently among populations. Indeed, the response to clipping in maximum inflorescence height showed opposite directions among populations in all regions except CSA (Fig. 4.2) and such opposing responses to clipping are also visible in some other traits. Seed families were differentiated

but responded similarly to the treatment (Table 4.1), 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 circa six months old when transplantated to the common garden and most traits were measured >2.5 months after transplantation. The effect of initial rosette diameter on phenotypic traits indicate that small initial size differences in *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. Such initial size differences may be partly due to maternal effects, suggesting that the influence of maternal effects diminish over time, as has been found in other studies (e.g. Ouborg et al. 1991; Schmid and Dolt 1994). Therefore we assume that maternal effects are negligable, especially for reproductive traits which are related most strongly to fitness. It could be argued that initial maternal effects can be propagated over time through unequal intra- and interspecific competition. However, we regularly weeded the common garden and provided ample space between experimental plants, which limits the enhancement of initial phenotypic differences.

Correlations

Correlations between altitude of origin and number of inflorescences, maximum inflorescence height and above-ground biomass measured in the common garden were significant and negative (Table 4.3). This suggests 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 and Renhardt 1987), but the functional explanation of this pattern is still discussed. The inflorescence of *C. thyrsoides* determines both height and the larger part of the above-ground biomass. Therefore our results suggest that the widely-observed pattern in forbs may also apply to the inflorescence of monocarpic species and is at least partly genetic. The three traits correlating with altitude are clearly also correlated with each other, reflecting a decreased energy budget with increasing altitude. Interestingly, number of flowers did not decrease with altitude, suggesting that the observed patterns are a result of adjusted architecture.

The correlations could be due to regional effects and not to local adaptation to altitude. In this study, all SEA plants occur at much lower altitude compared to plants from the other regions, and this 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). However, removing SEA from the analysis did not change the direction of the significant correlations although the strength decreased (Table 4.3), indicating that the relationships hold across subspecies with contrasting ecological requirements.

The comparably stronger correlations, found when any effects of phylogeographic region were additionally removed, are even better indicators of alpine-wide fine-grained local adaptation to environmental conditions related to altitude. Monty and Mahy (2009) found similar negative relationships for final height and above-ground biomass in a common garden experiments 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).

Heritability and Q_{ST}-G'_{ST} analysis—Average heritability of all traits was substantial (Table 4.4), but about half of the populations lacked heritability, limiting the possibility for change through natural selection. Q_{ST} of all traits except above-ground biomass was smaller than the bias-corrected G'_{ST_est}, sug-

gesting unifying selection among populations. Unifying selection has also been found for specific leaf area in the same species (Scheepens et al. 2010). Adaptation therefore possibly restricted differentiation by genetic drift and mutations. Comparing Q_{ST} to D_{est} would result in a similar conclusion, but the opposite conclusion would be drawn based on a comparison of Q_{ST} and G_{ST_est}. The debate which differentiation index to use is still ongoing and is focussing on the relative strength of mutation and migration (Jost 2008; Whitlock 2011). G_{ST_est} and related indices such as F_{ST} have been shown to largely underestimate differentiation when multiple alleles are present (Jost 2008), as in the current data (Ægisdóttir et al. 2009; Kuss et al. 2011), and G'_{ST_est} and D_{est} were designed to correct for this effect, although they might overestimate the true differentiation (Whitlock 2011). We can assume that among the isolated populations of *C. thyrsoides* migration rates are much lower than mutation rates, in which case the overestimation by G'_{ST_est} and D_{est} is relatively weak or absent (Whitlock 2011) and we can thus conclude that unifying selection played a role in shaping phenotypic differentiation.

Conclusions

We showed that phenotypic differentiation among phylogeographic lineages is in line with neutral molecular differentiation. We suggest that glacial history is responsible for this phenotypic differentiation and that the four phylogeographic lineages diverged independently by drift and selection in the past and present. Q_{ST}-G'_{ST} analysis indicated that past selection pressures played a role in shaping the phenotypic differentiation among populations, but drift could have played an important role as well throughout glacial history. Trait-altitude correlations suggested that number of inflorescences, maximum inflorescence height and above-ground biomass are adapted to altitude. Current selection regimes in the heterogeneous landscape of the Alps may therefore also play an important role in local adaptation, as is also suggested by the larger variance explained by populations compared to regions. There were also indications for current adaptation with a regional pattern. First, the flowering behaviour in SEA populations is strongly adapted to the submediterranean climate; second, WA plants exhibited low susceptibility to grazing, which is in accordance with its predominant occurrence in pastures and meadows. Postglacial adaptation was indicated by the populations from the Jura Mts. showing clear phenotypic differentiation from other WA populations despite absence of neutral molecular differentiation in this region. This pattern is likely due to adaptation of Jura populations to specific environmental conditions at lower altitude and in different vegetation communities. We conclude that glacial history caused part of the observed phenotypic differentiation. Although recent adaptation to the heterogeneous environment is probably a stronger effect, these adaptations can be structured according to phylogeography, and thus be ultimately an effect of glacial history.

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Appendix

Table 4.5: Location, geographic coordinates (WGS 84) and altitude (m a.s.l.) of 21 sampled *Campanula thyrsoides* populations across the Alps and Jura Mts. 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.

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

Table 4.6: Results of GLMM analysis of the effects of initial rosette diameter (covariate), distance to common garden (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. See Methods section for details on GLMMs. Chi^2 values and their significancies were obtained from model comparisons. *%VC*—Variance components were obtained from analyses with all factors treated as random effects. df—degrees of freedom, residual df varies per trait due to mortality and due to flowering traits being recorded in flowering plants only.

	Post-transplantation survival				Number of leaves			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	479.0	***	62.8	1	156.7	***	16.3
Distance to garden	1	0.1		2.6	1	23.2	***	3.6
Clipping treatment	1	-		-	1	150.5	***	23.5
Region	3	22.6	***	18.3	3	44.3	***	2.6
Population (Region)	1	0.8		2.6	1	67.3	***	3.6
Seed family (Population)	1	0.7		0.5	1	4.0	*	2.2
Clipping×Region	3	-		-	3	4.4		0.0
Clipping×Pop (Region)	1	-		-	1	0.1		0.4
Clipping×Seed family (Pop)	1	-		-	1	0.5		2.8
Residuals	942			13.1	789			44.9
	Leaf length to width ratio 2008				Leaf length to width ratio 2009			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	94.3	***	4.0	1	52.1	***	3.3
Distance to garden	1	1.5		8.9	1	2.0		3.7
Clipping treatment	1	1.1		0.2	1	36.6	***	6.4
Region	3	48.3	***	9.0	3	14.8	**	0.0
Population (Region)	1	108.9	***	8.9	1	49.4	***	3.7
Seed family (Population)	1	19.4	***	8.4	1	9.2	**	7.9
Clipping×Region	3	2.4		0.0	3	2.2		0.0
Clipping×Pop (Region)	1	0.1		0.8	1	4.4	*	5.5
Clipping×Seed family (Pop)	1	0.0		0.0	1	0.0		0.0
Residuals	790			60.0	755			69.4
	Number of inflorescences				Maximum inflorescence height			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	15.4	***	0.1	1	52.5	***	0.0
Distance to garden	1	27.7	***	1.9	1	71.0	***	6.6
Clipping treatment	1	52.1	***	1.6	1	20.7	***	2.7
Region	3	52.2	***	1.0	3	103.0	***	23.9
Population (Region)	1	35.7	***	1.6	1	46.4	***	6.5
Seed family (Population)	1	16.0	***	2.3	1	18.1	***	9.7
Clipping×Region	3	1.8		0.0	3	7.7	(*)	0.0
Clipping×Pop (Region)	1	0.0		0.0	1	7.1	**	5.3
Clipping×Seed family (Pop)	1	0.0		0.0	1	0.0		0.0
Residuals	672			91.5	672			45.4
	Number of flowers				Above-ground biomass			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	34.9	***	0.3	1	85.1	***	10.4
Distance to garden	1	3.3		4.0	1	36.8	***	1.9
Clipping treatment	1	95.9	***	21.5	1	76.5	***	14.8
Region	3	41.3	***	0.8	3	55.7	***	15.9
Population (Region)	1	18.2	***	5.9	1	10.5	**	1.9
Seed family (Population)	1	0.0		1.6	1	4.6	*	4.7
Clipping×Region	3	7.7	(*)	1.0	3	18.6	***	3.0
Clipping×Pop (Region)	1	0.0		1.9	1	1.1	***	1.8
Clipping×Seed family (Pop)	1	0.0		0.0	1	0.0		0.0
Residuals	609			62.9	681			45.5

(*) $P = 0.054$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4.7: Results of GLMM analysis of the effects of initial rosette diameter (covariate), altitude of origin (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. See Methods section for details on GLMMs. Chi^2 values and their significancies were obtained from model comparisons. *%VC*—Variance components were obtained from analyses with all factors treated as random effects. df—degrees of freedom, residual df varies per trait due to mortality and due to flowering traits being recorded in flowering plants only.

	Post-transplantation survival				Number of leaves			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	479.0	***	62.5	1	156.7	***	16.3
Altitude of origin	1	0.5		2.7	1	18.7	***	3.6
Clipping treatment	1	-		-	1	149.7	***	23.5
Region	3	20.8	***	18.4	3	68.8	***	2.6
Population (Region)	1	1.2		2.7	1	51.1	***	3.6
Seed family (Population)	1	0.8		0.5	1	4.1	*	2.2
Clipping×Region	3	-		-	3	4.4		0.0
Clipping×Pop (Region)	1	-		-	1	0.1		0.4
Clipping×Seed family (Pop)	1	-		-	1	0.5		2.8
Residuals	942			13.2	789			44.9
	Leaf length to width ratio 2008				Leaf length to width ratio 2009			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	94.3	***	4.0	1	52.1	***	3.3
Altitude of origin	1	24.9		8.8	1	2.7		3.7
Clipping treatment	1	1.3		0.2	1	36.5	***	6.4
Region	3	38.8	***	9.0	3	49.9	**	0.0
Population (Region)	1	97.0	***	8.8	1	23.2	***	3.7
Seed family (Population)	1	19.2	***	8.4	1	8.9	**	7.9
Clipping×Region	3	2.4		0.0	3	2.1		0.0
Clipping×Pop (Region)	1	0.1		0.8	1	4.4	*	5.5
Clipping×Seed family (Pop)	1	0.0		0.0	1	0.0		0.0
Residuals	790			60.0	755			69.3
	Number of inflorescences				Maximum inflorescence height			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	15.4	***	0.1	1	52.5	***	0.0
Altitude of origin	1	69.8	***	1.9	1	139.2	***	6.6
Clipping treatment	1	48.6	***	1.6	1	20.2	***	2.7
Region	3	46.8	***	1.0	3	34.0	***	23.9
Population (Region)	1	0.0	***	1.6	1	46.5	***	6.5
Seed family (Population)	1	36.2	***	1	2.3	19.3	***	9.7
Clipping×Region	3	1.8		0.0	3	7.8	(*)	0.0
Clipping×Pop (Region)	1	0.0		0.0	1	6.7	**	5.3
Clipping×Seed family (Pop)	1	0.0		0.0	1	0.0		0.1
Residuals	672			91.5	672			45.4
	Number of flowers				Above-ground biomass			
	df	Chi^2		*%VC*	df	Chi^2		*%VC*
Initial rosette diameter	1	34.9	***	0.3	1	85.1	***	10.4
Altitude of origin	1	3.8	(*)	4.0	1	82.6	***	1.9
Clipping treatment	1	92.5	***	21.5	1	81.5	***	14.8
Region	3	78.9	***	0.8	3	31.7	***	15.9
Population (Region)	1	0.0	***	5.9	1	0.1	**	1.9
Seed family (Population)	1	0.4		1.6	1	4.4	*	4.7
Clipping×Region	3	8.1	*	1.0	3	19.0	***	3.0
Clipping×Pop (Region)	1	0.1		1.9	1	0.4		1.8
Clipping×Seed family (Pop)	1	0.1		0.0	1	0.0		0.0
Residuals	609			62.9	681			45.6

(*) $P = 0.054$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Chapter 5

Differentiation in morphology and flowering phenology between two *Campanula thyrsoides* L. subspecies

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Differentiation in morphology and flowering phenology between two *Campanula thyrsoides* L. subspecies

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Abstract

Subspecies are usually characterized by sets of morphological discontinuities. By means of common garden experiments, we investigated genetic differentiation in morphological and phenological traits in two geographically disjunct subspecies of *Campanula thyrsoides* L., i.e. subsp. *thyrsoides* (= *C.* thyrsoides*) occurring in the European Alps and Jura Mountains, and subsp. *carniolica* (= *C.* carniolica*) occurring in the Southeastern Alps and the Dinaric Arc. Nine out of 16 investigated traits were significantly different between *C.* thyrsoides* and *C.* carniolica*. For *C.* carniolica* inflorescence length was 1.4×, and above-ground biomass 2.7× higher, while flower density was significantly lower. *Campanula* carniolica* also showed delayed flowering and flower development from bottom to top compared to *C.* thyrsoides* which flowered from top to bottom. The inflorescence growth was indeterminate and flowering took several weeks in *C.* carniolica*, whereas *C.* thyrsoides* showed determinate flowering, rapidly opening all flowers within a few days. This differentiation in flowering phenology is likely to be adaptive. The submediterranean climate favours indeterminate flowering in *C.* carniolica*, allowing ongoing growth of the inflorescence throughout the long summer until environmental conditions worsen, whereas determinate and early flowering in *C.* thyrsoides* is favourable in the short growing season in the high Alps where seed production must be secured before temperature drops. Glacial survival in refugia with different climates (alpine vs. submediterranean) may have caused this regional differentiation.

Keywords: *Campanula thyrsoides* subsp. *carniolica*; common garden; determinate flowering; European Alps; glacial history

Introduction

Subspecies are usually characterized by sets of morphological discontinuities but incomplete reproductive isolation (Stuessy 1990). The European Alps are notably rich in endemics, whether at species or subspecies level (Aeschimann et al. 2004), suggesting that speciation rate is high compared to the surrounding lowland. Glacial history has likely played a major role in taxon differentiation within the European Alpine flora, due to lineage divergence during survival in isolated refugia (Comes and Kadereit 2003; Tribsch and Schönswetter 2003; Paun et al. 2008). Ad-

ditionally, the spatial heterogeneity of the Alps in terms of topography, geology and regional climate may have caused local or regional adaptation of the various lineages (Alvarez et al. 2009; Stöcklin et al. 2009; Scheepens et al. 2010; Winkler et al. 2010). The last decade experienced a wave of studies investigating differentiation of Alpine species with a focus on glacial history as the driver of neutral genetic differentiation within and among taxa (Schönswetter et al. 2005; Gugerli et al. 2008). While strong genetic barriers between intraspecific groups of populations have been widely detected, these molecular studies usually do not extend to differentiation at the phenotypic level and thus may overemphasize the role of neutral processes in differentiation of related taxa (but see Paun et al. 2008; Winkler et al. 2010). In this study on *Campanula thyrsoides*, we hypothesize the presence of glacial history-related phenotypic differentiation between the subspecies in traits observed in two common gardens and we discuss whether these patterns could be due to adaptation.

Campanula thyrsoides L. (Campanulaceae) is a monocarpic, yellow-flowering bell flower occurring predominantly on calcareous grassland slopes from 1600 to 2200 m a.s.l. in the European Alps (Kuss et al. 2007). Seeds have a low dispersal capacity and populations are rare and isolated but sometimes harbour high numbers of individuals, most of them as rosettes and some conspicuously flowering (Kuss et al. 2007). Within-population genetic diversity is high and among-population differentiation is substantial (Kuss et al. 2008a; Ægisdóttir et al. 2009). Its distribution ranges across the European Alps and the Jura Mts. with patchy occurrences in the Dinaric Arc (Kuss et al. 2007), but its occurrence is sparse in the Dolomites and Tyrol. To the west of this distribution gap the subspecies *Campanula thyrsoides* subsp. *thyrsoides* (*C.* thyrsoides*) occurs, and to the southeast of this gap, in the Southeastern Alps and the Dinaric Arc, the majority of populations can be classified to the other subspecies, *Campanula thyrsoides* subsp. *carniolica* (*C.* carniolica*). It is unknown if the two subspecies are interfertile and produce viable offsprings.

The Southeastern Alpine subspecies *C.* carniolica* was first described as a variety by Sündermann (1925) who observed it to be taller than *C.* thyrsoides* due to an elongated inflorescence. Flower density was reported to be more lax in the lower part of the inflorescence compared to *C.* thyrsoides*, and its bracts were found to be up to double the length of those of *C.* thyrsoides*. The time of flowering was observed to be delayed for *C.* carniolica* compared to *C.* thyrsoides*, and Jäger (2000) observed that *C.* thyrsoides* flowered in July and August and *C.* carniolica* in the first half of August, thus partly overlapping in flowering. The varieties were ranked as subspecies by Podlech (1964) based on the marked difference in their geographical distribution in addition to the morphological and phenological characteristics described above: in contrast to the alpine to subalpine *C.* thyrsoides*, *C.* carniolica* occurs at submontane to montane elevations (400-1800 m a.s.l.) and is confined to the Carnic area in the broad sense, which includes parts of Italian Carnia and Slovenia (Carnian and Julian Alps) as well as Austrian Carinthia (Jäger 2000; Kuss et al. 2007 and references therein). It has been speculated that these two subspecies are altitudinal vicariants (Tomazic 1941) though the elongated inflorescence of *C.* carniolica* may also be the result of adaptation to the submediterranean climate (Kuss et al. 2007).

Jäger (2000) suggested that the geographic partitioning might be a result of glacial history, the two subspecies having survived glaciated periods in different refugia on the fringes of the Alps. A microsatellite study investigating neutral genetic differentiation among 51 populations of *C. thyrsoides* across the Alps increased the understanding of the effects of glacial history on intraspecific differentiation (32 Swiss populations in Ægisdóttir et al. 2009; 51 populations in Kuss et al., unpublished results). The among-population genetic structure showed four spatial clusters across the Alps, which corresponded to the general phylogeographic pattern observed in several widespread Alpine species (Schönswetter et al. 2005; Alvarez et al. 2009; Thiel-Egenter et al. 2009). The most eastern cluster of these belonged to *C.* carniolica* and was clearly separated from the other three to the west, belonging to *C.* thyrsoides*. Analysis of molecular variance (AMOVA) showed that differentiation between the subspecies *C.* carniolica*

and *C.* thyrsoides* (i.e. the single eastern versus the three western clusters taken together) explained 8.4% of the variation. This was higher than differentiation between clusters within *C.* thyrsoides* and it was even higher than the partitioning of variation among all four distinct groups of populations, i.e. 6.7%. The genetic structure therefore supports the subspecies division of Podlech (1964) and is in line with Jäger's (2000) suggestion that glacial survival in separate refugia is at the origin of the subspecies division.

Morphological differences allow to identify the two subspecies in the field, but a study on their quantitative differences in various traits, however, has never been conducted. Botanists have sometimes found that presumed subspecies phenotypes are merely due to environmental differences, so it is important to be aware of environmental effects on phenotypic expression when taxonomy is concerned (Sultan 2000). To make sure that a quantitative analysis of morphological and phenological differences reflects genetic differences and not the influence of the environment, the subspecies can be grown together in a common garden (Clausen et al. 1948). The uniform environmental conditions in a common garden eliminate variation due to the environmental component, leaving only genetic differences (or rather genotype×environment expressions based on a single environment) between the subspecies to be observed (Connor and Hartl 2004).

In this study, we have chosen traits which are used to differentiate between the subspecies (inflorescence height, number of flowers per inflorescence length, flowering phenology) as well as traits which have not been investigated so far (e.g. leaf size, number of leaves, number of flowers, above-ground biomass). Specific leaf area (SLA) and leaf thickness have been measured because they are known to be differentiated between the subspecies (Scheepens et al. 2010). Since *C.* carniolica* generally occurs on roadsides and on rock outcrops, as opposed to *C.* thyrsoides* which mainly occurs in managed grasslands, it is likely that grazing regimes differ between the subspecies, which may have led to adaptive differentiation in response to grazing between the subspecies. Therefore, we assess the response to simulated herbivory in *C.* thyrsoides* and *C.* carniolica* observed in one of the two common garden experiments. Additionally, the common garden results are compared with field data to see if patterns of quantitative differentiation match between the common garden and the field. In summary, we ask (1) whether the subspecies *C.* thyrsoides* and *C.* carniolica* show glacial history-related differentiation in quantitative traits and (2) whether patterns of differentiation could be explained as adaptation to the subspecies' respective environments.

Methods

Experimental design

Two common garden experiments were performed in the Swiss Alps in order to obtain data on 16 quantitative traits. The seed sources of these experiments were populations which have been investigated as part of a phylogeographic study and for which spatial genetic information is available (Ægisdóttir et al. 2009; Kuss et al., unpublished results). The first experiment was located in Davos (1530 m a.s.l.) and herbivory was simulated by clipping half of the plants eight weeks after transplantation. This experiment included seed-derived plants from 17 populations of *C.* thyrsoides* and four populations of *C.* carniolica*. Six seed families per population and eight individuals per seed family made a total of 963 individuals at the beginning of the experiment (45 individuals were missing from the start).

The second experiment near Chur included three common gardens at different elevations along the slope of Mt Calanda, Switzerland (600, 1235, 1850 m a.s.l.). This experiment included seed-derived plants from 12 populations of *C.* thyrsoides* and six populations of *C.* carniolica*. A range of 2-7 (median 7) seed families per population and 2-12 (median 6) individuals per seed family added up to 600 individuals. For both expriments, seeds were germinated in the greenhouse during autumn 2007 and plants were transplanted to the common gardens of Davos and Chur in late spring and early summer 2008, respectively. In total, 15 out of 24 sampled populations were represented in both experiments. Blocks were initially incorporated in both common gardens, but these were never significant and therefore not considered in subsequent anal-

yses. Locations, altitudes and sample size of populations are given in Appendix Table 5.4. More detailed descriptions of the design of the Chur experiment are given by Scheepens et al. (2010).

Common garden measurements

Eight weeks after transplantation to Davos, survival was recorded and leaf thickness was measured (Teclock SM-112 dial thickness gauge, Okaya, Japan). Number of leaves was counted at the end of the growing season 2008. Length and width of longest leaf were measured at the end of the growing season 2008 as well as at the start of the growing season 2009. Reproductive traits were quantified halfway of the second growing season 2009, i.e. number of inflorescences, total inflorescence length, maximum inflorescence height, total number of flowers on all inflorescences, maximum number of flowers on an inflorescence, and number of flowers per inflorescence length (concerning the flower-bearing part of the inflorescence). Finally, when a plant had finished flowering in 2009, above-ground biomass was harvested, dried for 72 h at 60 °C, and weighed. Only data from unclipped plants were used (477 individuals), except for data on the response to the herbivory simulation.
Data on SLA and leaf hair density were obtained from the Chur experiment. Details on the measurement of SLA are described in Scheepens et al. (2010). Leaf hair density was measured per individual as the mean number of leaf hairs on five randomly chosen areas of 0.25 cm^2 on the top side of leaves. For SLA and leaf hair density, mean values for both subspecies were calculated via population means, which in turn were based on seed family means. For SLA, population averages were calculated for each elevational site, which were subsequently averaged over all three sites. Leaf hair density data was analysed without respect to altitudinal origin.
The response of selected traits to simulated herbivory was expressed as the proportional difference of clipped plants to control plants at the seed family level and averaged at the population level. A value of 1 thereby indicates absence of response in clipped plants relative to the control plants (i.e. full recovery after clipping). The three elevational sites in Chur were used to investigate the response of SLA to the elevation treatment, which was measured as the coefficient of variation at the population level and based on seed family averages.

Field measurements

The common garden results for inflorescence height, maximum number of flowers per inflorescence as well as number of flowers per inflorescence length were compared with those from field data obtained in 2006 from populations of both *C.* thyrsoides* and *C.* carniolica* in Slovenia, Italy and Austria. Percentage of withered flowers was estimated in these natural populations in order to investigate phenological differences between the subspecies. Here, the number of withered flowers was divided by the total number of flowers (including buds).

Data analysis

Subspecies comparisons were performed for all traits for both common garden and field data using non-parametric Mann-Whitney U tests, which do not rely on normality of the data and are more robust to outliers than Student's t-tests (Quinn and Keough 2002). We did not use any correction for multiple testing (e.g. sequential Bonferroni correction; Holm 1979), since most traits showed conspicuous though moderately significant differences between the subspecies, which would be eliminated by a multiple-testing correction (Moran 2003). All comparisons were based on population means, which in turn were based on seed family means for the common garden data. The coefficient of variation was calculated for each trait among populations within each subspecies, in the common garden as well as in the field, to investigate whether the two subspecies differed in variability and whether common garden and field sites differed in variability.

Results

Common garden measurements

Nine out of 16 traits were significantly different between *C.* thyrsoides* and *C.* carniolica* (Table 5.1). Post-transplantation survival did not differ significantly between the subspecies. Leaves were on average 10% thicker and SLA

was on average 14% lower in *C.* carniolica*. In fact, SLA was lower at all experimental elevations for *C.* carniolica* compared to *C.* thyrsoides* (data not shown, see Scheepens et al. 2010). Leaf hair density was 53% higher in *C.* carniolica* compared to *C.* thyrsoides*, but this difference was not significant. Rosettes of *C.* carniolica* had 25% fewer leaves. During the following year, flowering *C.* carniolica* plants had few rosette leaves or none at all, whereas flowering *C.* thyrsoides* plants showed a full (although withering) rosette (J.F. Scheepens, personal observation from both experiments). In both late and early season (i.e. measurements from the first and the second season, respectively), full-grown leaves were remarkably longer and wider in *C.* carniolica*, although width was not significantly different between the subspecies. The number of inflorescences did not differ statistically, but tended to be higher in *C.* carniolica*. The cumulative lengths of inflorescences was 1.8 times higher and the maximum inflorescence length was 1.4 times higher in *C.* carniolica*. The total number of flowers on all inflorescences as well as the maximum number of flowers on an inflorescence did not differ significantly between the subspecies, but tended to be lower in *C.* carniolica*. The number of flowers per length of inflorescence, however, was significantly lower for *C.* carniolica*. Finally, above-ground biomass was about 2.7 times higher in *C.* carniolica*.

Variation in trait values was considerably higher in *C.* carniolica* compared to *C.* thyrsoide*s (>5%; Table 5.1) for the following traits: leaf width at end of season, total number of flowers on all inflorescences and number of flowers per inflorescence length. By contrast, considerably lower variation (<5%) was found for leaf hair density, number of inflorescences and above-ground biomass.

Response to simulated herbivory was indifferent between subspecies for most of the selected traits: number of leaves, leaf length and width in both seasons, total inflorescence length and maximum inflorescence height showed comparable variability in the two subspecies (Table 5.2). *Campanula* carniolica* showed a significantly larger reduction in number of inflorescences after clipping (-15%; P=0.04) and a marginally significant larger reduction in above-ground biomass after clipping (-21%; P=0.07). *Campanula* carniolica* showed a less strong reduction in the number of flowers due to clipping compared to *C.* thyrsoides*, but this difference was not significant (+11%; P=0.26). The response of SLA to elevational treatments did not differ between the subspecies (mean ± se of population-level coefficient of variance (%): *C.* thyrsoides* 12.4 ± 1.0; *C.* carniolica* 12.3 ± 1.6; P=0.89).

Field measurements

The field data showed similar differences between the subspecies as did the common garden experiments, but the field and common garden data differed quantitatively (Table 5.1, 5.3). In natural populations, the inflorescence height was 1.7 times higher in *C.* carniolica* compared to *C.* thyrsoides*. Comparably, in the common garden of Davos the maximum inflorescence height was 1.4 times higher in *C.* carniolica* (Table 5.1). Whereas the relative differences were similar between the subspecies, the average heights of 24 and 40 cm for *C.* thyrsoides* and *C.* carniolica* in the field were smaller than in the common garden, where average heights of 39 and 54 cm were observed, respectively. The number of flowers did not differ between the two subspecies in the natural populations, in accordance with the results from the common garden. The number of flowers per inflorescence length was 30% lower in *C.* carniolica* both in the field and the common garden, but the absolute values in the field were smaller than in the common garden. The percentage of withered flowers was only measured in the field and was significantly lower for *C.* carniolica* than for *C.* thyrsoides*, since populations from the latter finished flowering already at the time of measurement (Table 5.3).

Table 5.1: Mean, standard error (se), and coefficient of variance (cv) for 16 traits of two subspecies of *Campanula thyrsoides* L., as well as quantitative differences between species (%) and significance levels (P) based on Mann-Whitney U tests.

	Campanula thyrsoides*			*Campanula* carniolica*				
	mean	se	cv(%)	mean	se	cv(%)	%	P
Survival (proportion)	0.88	0.05	22.2	0.77	0.09	22.5	-12.5	0.33
SLA (cm^2g^{-1})	160.9	2.4	5.1	137.8	3.9	6.9	-14.3	<0.001
Leaf thickness (mm)	0.486	0.006	5.1	0.534	0.021	7.9	9.9	<0.05
Leaf hair density (cm^{-2})	15.2	3.1	70.3	23.3	4.7	49.6	53.3	0.18
Number of leaves	131.2	6.8	21.2	97.5	10.7	22.0	-25.7	<0.05
Leaf length end of season (cm)	7.20	0.24	13.8	9.92	0.87	17.5	37.8	<0.01
Leaf width end of season (cm)	2.65	0.08	12.5	3.31	0.37	22.6	24.9	0.08
Leaf length start of season (cm)	11.3	0.4	14.6	14.1	1.0	13.9	24.8	<0.01
Leaf width start of season (cm)	1.58	0.05	12.7	1.92	0.16	16.8	21.5	0.07
Number of inflorescences	3.80	0.40	43.2	4.69	0.31	14.0	23.4	0.41
Total inflorescence length (cm)	176	14	31.7	317	52	32.6	80.1	<0.05
Maximum inflorescence height (cm)	39.2	1.3	14.0	54.3	3.8	14.2	38.5	<0.01
Total number of flowers on all inflorescences	348	25	29.8	276	70	50.8	-20.7	0.32
Maximum number of flowers on inflorescence	102.8	4.3	17.1	86.0	9.2	21.6	-16.3	0.17
Number of flowers per inflorescence length (cm^{-1})	6.67	0.13	8.2	4.96	0.44	17.9	-25.6	<0.001
Above-ground biomass (g)	25.7	2.5	40.4	69.5	5.0	14.4	170.4	<0.001

Table 5.2: Response to simulated herbivory of two subspecies of *Campanula thyrsoides* L. Given are mean, standard error (se) and coefficient of variance (cv) in ten traits, as well as quantitative differences between subspecies (%) and significance levels (P) based on Mann-Whitney U tests.

	Campanula thyrsoides*			*Campanula* carniolica*				
	mean	se	cv(%)	mean	se	cv(%)	%	P
Number of leaves	0.82	0.02	9.7	0.83	0.03	7.8	0.78	1.00
Leaf length end of season	0.85	0.01	6.6	0.83	0.03	7.4	-3.20	0.44
Leaf width end of season	0.95	0.02	5.2	0.91	0.03	3.5	-4.48	0.34
Leaf length start of season	0.84	0.01	8.7	0.81	0.01	6.9	-3.70	0.18
Leaf width start of season	0.99	0.01	5.4	0.98	0.03	5.2	-1.16	0.56
Number of inflorescences	0.91	0.03	13.7	0.77	0.02	5.0	-14.79	<0.05
Total inflorescence length	0.88	0.04	18.8	0.86	0.06	14.8	-2.86	1.00
Maximum inflorescence height	0.99	0.02	9.5	0.95	0.04	9.5	-4.05	0.75
Total number of flowers	0.79	0.04	19.3	0.87	0.06	14.0	10.85	0.26
Above-ground biomass	0.84	0.07	34.2	0.66	0.02	5.4	-21.10	0.07

Phenology

In the common garden in Davos, *C.* carniolica* showed a delayed flowering compared to *C.* thyrsoides*. At the start of the second season (1 June 2009) on average 89% of surviving *C.* thyrsoides* plants had already started bolting, whereas *C.* carniolica* plants showed no signs of bolting yet. On 27 July 2009, 93% of *C.* thyrsoides* plants had finished flowering and were in their seed ripening stage and ready to be harvested, whereas 83% of *C.* carniolica* plants were now fully flowering and 7% ripening. Harvesting of *C.* carniolica* took place on 20 October 2009 when snow and frost were abound. However, *C.* carniolica* plants were still flowering or had just started ripening, indicating that they were not adapted to the short growing season at this elevation.

Inflorescences of *C.* carniolica* flowered from the bottom to the top, whereas *C.* thyrsoides* flowered from the top to the bottom. More

specifically, flowering in *C.* carniolica* started where the lowest flowers were still closely connected to the inflorescence stem, thus without pronounced secondary stem formation. Below this point secondary stems usually occured, each with multiple flowers which opened later as well. Flowering was indeterminate in *C.* carniolica*, so new buds were continuously being formed at the apex, and anthesis from bottom to top took several weeks. Consequently, fruits in different stages of ripening could be found below the open flowers. This is in contrast to *C.* thyrsoides* which showed determinate flowering from the top, rapidly opening all flowers within a few days.

Figure 5.1 shows the most pronounced differences in the habitus of the two subspecies, including for *C.* carniolica* (i) the taller inflorescence, (ii) the smaller number of rosette leaves in the flowering individual, (iii) the lax flower positioning, (iv) the thinner inflorescence and (v) the indeterminate flowering phenology.

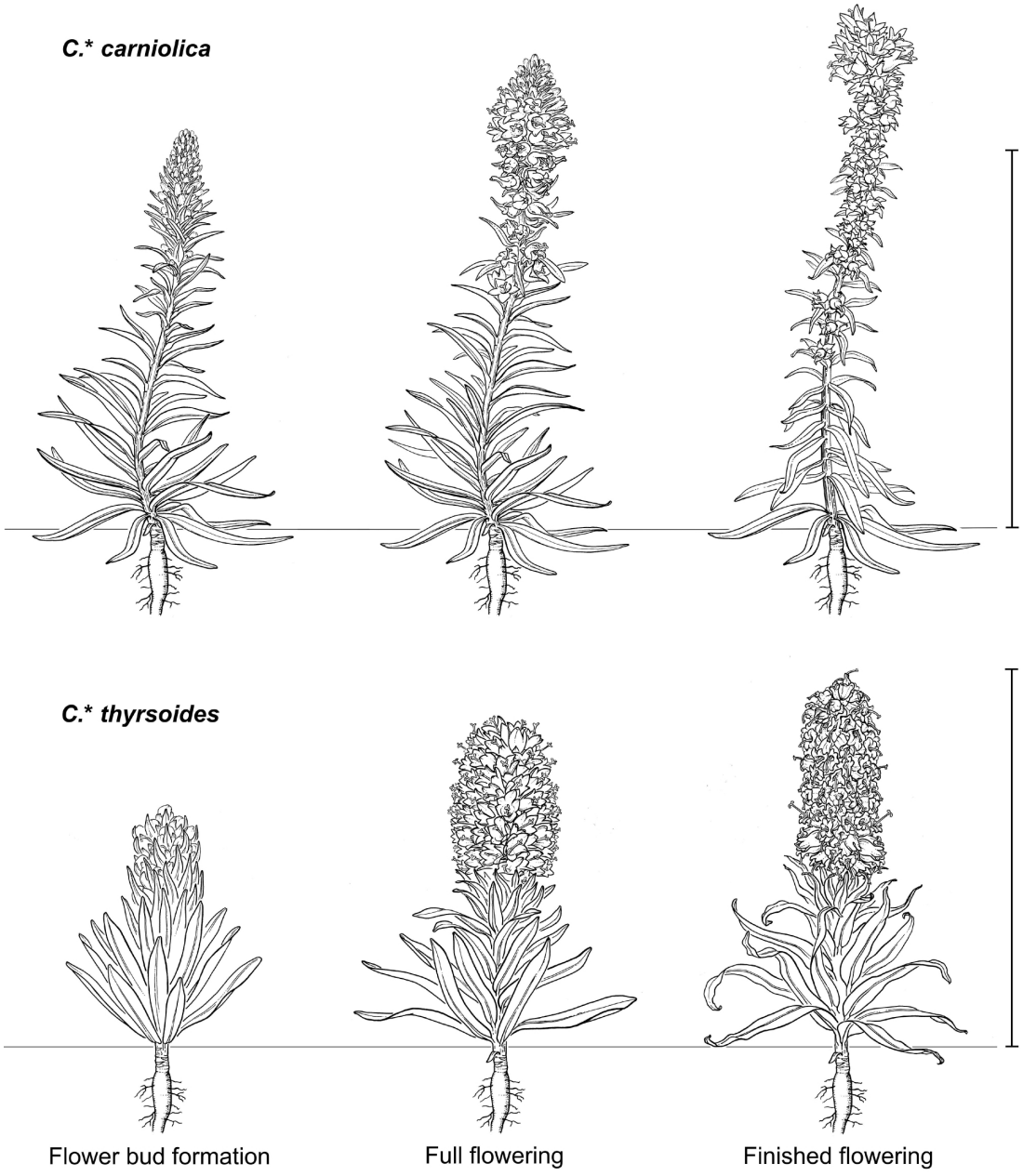

Figure 5.1: Habitus of *Campanula thyrsoides* subsp. *carniolica* and subsp. *thyrsoides* in three phenological phases of flowering. Scale bars have the same length, indicating the relative size of the two subspecies. Drawing by Atelier Guido Köhler, Basel

Discussion

The common garden data support the subspecies division in *Campanula thyrsoides* since clear differences were observed between the two subspecies in the majority of the examined traits, among them traits used to distinguish the subspecies taxonomically such as inflorescence height (Table 5.1). Although we did not correct for multiple-testing, obtaining $P<0.05$ for nine out of 16 traits by chance has a probability of $<1.6\times10^{-8}$ based on a Bernoulli process (Moran 2003). The morphological and phenological differences observed in the field were also observed in the common garden indicating genetic differences: *C.* carniolica* showed a taller inflorescence height, a more lax flower positioning and delayed flowering (Table 5.1, 5.3). In addition, the similar number of flowers in the two subspecies was observed in the common garden as well as in the field (Table 5.1, 5.3). Although flower number was similar between subspecies, the advantage of a taller inflorescence in *C.* carniolica* may lie in farther seed dispersal (Tackenberg et al. 2003; Tackenberg and Stöcklin 2008). Lower SLA and higher leaf thickness in *C.* carniolica* have been explained as adaptation to drought in the submediterranean climate (Scheepens et al. 2010). Length and width of leaves showed that start-of-season leaves were more elongate than end-of-season leaves, which is in accordance with observations by Jäger (2000) who described the two-phased rosette growth of *C. thyrsoides*.
Maternal effects have often been reported to affect a variety of traits, but maternal effects are usually pronounced at the seedling stage while decreasing over time (e.g. Ouborg et al. 1991; Schmid and Dolt 1994). Since plantlets were circa six months old at transplantation and since most traits were measured 2.5 months or more after transplantation, we assume that maternal effects are negligable, but cannot verify this. Initial maternal effects can be propagated through unequal intra- and interspecific competition, but a weeded common garden with ample space between experimental plants, as in our study, limits the enhancement of initial phenotypic differences. Genotype×environment effects were likely to be small in our study, as traits that were measured in both common gardens (comparison not shown) and in the field showed similar patterns.

Common garden versus field measurements

The common garden and field data showed that observations from experimental and natural settings can diverge. This is most evident from the number of inflorescences: common garden-grown individuals were predominantly multi-stemmed (90% in Davos, data not shown) in contrast to predominantly single-stemmed individuals under natural field conditions (88%, data not shown). It was most likely the benign conditions in the common garden, due to weeding and nutritious soil, which caused the growth of multiple inflorescences. Another notable difference between plants in the common garden and wild individuals is that the latter are reported to flower once after 3-16 years with an average of about ten years (Kuss et al. 2008b), whereas the vast majority (93%) of the surviving experimental plants in Davos flowered already in their second year. Comparing the common garden results with the field observations also showed smaller inflorescence height, number of flowers and flowers per inflorescence length in the field. To conclude, on the one hand common gardens are an ideal method to detect and compare genetic differences between subspecies, whereas on the other hand the obtained values cannot be used as descriptors to identify the subspecies in the field.

Coefficient of variation

Knowledge on intraspecific variability in plant traits is important as it could inform us about differences in niche breadth among taxa (Rotundo and Aguiar 2008; Milla et al. 2009; Albert et al. 2010). We found that coefficients of variation were remarkably similar for both subspecies in most traits, notably in those traits which differed significantly in their average values. Exceptions were number of flowers per inflorescence length and above-ground biomass, which differed both in their mean and coefficient of variation. This result indicates that variability in most traits among populations did not differ between the subspecies and may suggest that niche breadths of the two subspecies are similar. This is an interesting result, suggesting that

Table 5.3: Locations and means of four traits in two subspecies of *Campanula thyrsoides* L. from field data. Given are population-level mean±se or percentage, subspecies mean±se and coefficient of variance (cv), as well as quantitative differences between subsspecies (%) and significance levels (P) based on Mann-Whitney U tests. Subsp. *thyr.*: *C. thyrsoides* subsp. *thyrsoides*; *carn.*: *C. thyrsoides* subsp. *carniolica*

Population (number of individuals	Subsp.	North	East	Elevation (m a.s.l.)	Inflorescence height (cm)	Number of flowers	Flowers per cm inflorescence	% flowers withered
Rüfikopf (20)	*thyr*	47°12'00"	10°10'08"	2307	25.6±1.2	69.4±4.9	4.6±0.2	100
Elmer Kreuzspitze (7)	*thyr*	47°20'41"	10°34'34"	1874	22.3±0.9	43.8±5.9	4.7±0.5	100
Rinnen (13)	*thyr*	47°24'23"	10°42'46"	1215	23.3±1.2	28.7±2.7	3.4±0.2	100
Hintertux (15)	*thyr*	47°06'46"	11°39'05"	2011	25.3±2.3	58.9±6.3	4.9±0.4	89
				mean±se	*24.1±0.3*	*50.2±8.9*	*4.4±0.3*	*97.3±2.8*
				cv	*6*	*35*	*15*	*6*
Passo di Monte Croce Carnico (3)	*carn*	46°36'02"	12°57'05"	1629	33.7±1.8	47.7±6.4	2.8±0.4	72
Sella Nevea[a,b] (7)	*carn*	46°23'35"	13°27'46"	932	34.3±4.0	45.0±6.1	3.6±0.3	47
Plesa (4)	*carn*	46°17'55"	13°58'54"	950	38.5±9.8	49.5±12.1	4.6±0.2	57
Nemski Rovt[a,b] (11)	*carn*	46°23'35"	13°58'89"	663	23.1±3.3	46.0±4.2	3.2±0.2	63
Postojna (19)	*carn*	45°49'37"	14°14'25"	515	27.1±1.8	32.7±2.2	2.5±0.2	34
Sklendrovec[b] (22)	*carn*	46°06'22"	14°56'56"	339	49.6±2.2	57.6±3.2	2.8±0.1	93
Brodnice[a,b] (16)	*carn*	46°06'24"	15°16'53"	283	56.3±4.3	100.9±21.2	3.0±0.4	90
Vitanje[a,b] (18)	*carn*	46°22'27"	15°17'16"	422	55.4±3.2	57.9±4.3	2.5±0.3	75
				mean±se	*39.8±4.5*	*54.7±7.2*	*3.1±0.2*	*66.6±7.2*
				cv	*32*	*37*	*22*	*31*
				% difference	*65*	*9*	*-30*	*-31*
				P (thyr. vs. carn.)	*<0.05*	*0.93*	*<0.05*	*<0.05*

[a] Population also used in the common garden experiment in Davos;
[b] Population also used in the common garden experiment near Chur (see Scheepens et al. 2010).

trait variability is inherently constant (relative to the average) whereas average trait values can shift according to environmental conditions.
Another exception to the general pattern was the number of flowers in the common garden, which showed a much higher coefficient of variation for *C.* carniolica*. This high variability could be due to the uncommon environment for this subspecies. The number of flowers at harvesting was probably largely dependent on time of flowering in this indeterminately flowering subspecies, with early-flowering individuals yielding more flowers at harvesting than late-flowering individuals, resulting in high variability. In line with this argumentation, the coefficient of variation for the number of flowers of individuals in the field was similar for both subspecies.
Coefficients of variation showed a large range across traits, indicating that some traits (e.g. maximum inflorescence height) were more stable across populations and would therefore be more reliable as taxonomic indicators than others (e.g. biomass). Stronger environmental influences would be expected in the field, leading to increased variability. However, field measurements did not systematically show a higher coefficient of variation than the common garden measurements for the same traits.

Herbivory

Compensatory growth following herbivory is a general phenomenon in plants, but its extent can differ inter- and intraspecifically (Strauss and Agrawal 1999). This study showed that compensation in *C. thyrsoides* was generally strong and that the response to the herbivory simulation did not differ between the subspecies for most traits (Table 5.2), suggesting either (a) that any contrasting herbivory pressure between the habitats of the two subspecies did not lead to genetic differentiation in these traits or (b) that the two habitats did not differ in herbivory pressure. Suzuki (2008) similarly found no differences in plasticity in response to clipping in a common garden experiment among three populations of the annual *Persicaria longiseta* with different long-term deer grazing histories, and Rotundo and Aguiar (2008) likewise observed similar tolerance to defoliation among three populations of *Poa ligularis* differing in recent sheep grazing histories, although other studies do report differences among varieties or closely-related species in response to herbivory simulation (Welter and Steggal 1993; Westberg et al. 2010). It may be that herbivory thresholds, above which plant performance is differentially affected in the subspecies, have not been reached in our experiment (Strauss and Agrawal 1999).

Phenology

Differences in phenology between the subspecies may be hypothesised based on the contrasting environments they inhabit. As stated in the introduction, Sündermann (1925) reported *C.* carniolica* to flower later than *C.* thyrsoides*, and Jäger (2000) found that *C.* thyrsoides* reached full flowering between July and August whereas *C.* carniolica* reached full flowering in the first half of August. In line with these observations, *C.* carniolica* flowered later than *C.* thyrsoides* in our common gardens. However, the phenology as recorded from the common garden experiment must not necessarily represent the phenology under natural conditions, since the delayed flowering of *C.* carniolica* may be the result of a genotype×environment interaction. From the literature it is known that, together with photoperiod and moisture, temperature is a major cue to flowering, usually observed in common garden experiments using plants from different latitudes (Rathcke and Lacey 1985; Weber and Schmid 1998; Olsson and Ågren 2002). If there would be a temperature threshold as cue to flowering in *C.* carniolica*, this should then have occurred much later in the season at the high elevation of the common garden in Davos. The field observations were in agreement with the observed delayed flowering in the common garden, showing 97% of flowers of *C.* thyrsoides* plants withered and 67% of flowers of *C.* carniolica* withered (Table 5.3). It should be noted that three of the four *C.* thyrsoides* populations were cencused one month later than all other populations in the field study. However, the Hintertux population of *C.* thyrsoides* was measured at the same time as the *C.* carniolica* populations and had also finished flowering (89% withered), thereby contrasting with *C.* carniolica* populations. Thus, the subspecies differ in phenology,

with *C.* carniolica* showing delayed flowering. Strictly speaking, that *C.* thyrsoides* plants in natural populations were already ripening when *C.* carniolica* was still flowering in Slovenia indicates an earlier end of flowering in *C.* thyrsoides* and not per se advanced flowering initiation. Nevertheless, delayed start of flowering at lower elevation for *C.* carniolica* fits well with the phenology of alpine versus lowland populations, reflecting the different geographical distributions of the two subspecies. Advanced flowering at higher elevations could be explained as adaptation to the short growing season during which the plant needs to fulfill its life-cycle (Kudo 1993; Olsson and Ågren 2002; Sandring et al. 2007). Thus, the delayed flowering of *C.* carniolica* in Slovenia's relatively low mountains could be explained as adaptation to the submediterranean climate of this area, but more evidence is needed to strengthen this claim. We speculate that this phenological mismatch between the two subspecies as observed in a common environment as well as in the field could essentially entail reproductive isolation, which is a key driver of speciation (Coyne and Orr 2004).
It could be hypothesised that the indeterminate flowering in *C.* carniolica* versus the determinate flowering in *C.* thyrsoides* is due to adaptation to climate. The submediterranean climate could favour indeterminate flowering in *C.* carniolica*, because this would allow a longer flowering period throughout the long summer until environmental conditions would deteriorate, whereas determinate and fast flowering would be more favourable in the short growing season in the high Alps where seed production must be secured before temperatures drop.
Our common garden data also showed that *C.* thyrsoides* populations reached full flowering simultaneously. The same was true for *C.* carniolica* populations, which flowered simultaneously but later than *C.* thyrsoides*. If it is true that natural populations of *C.* thyrsoides* have a wide range in peak flowering, as is suggested by Jäger's (2000) broad range in flowering time, our data would then suggest a plastic response of flowering to climatic factors, where the common environment in Davos led to synchronous flowering. It could therefore be hypothesised that, although flowering phenology is genetically differentiated between the subspecies, climatic factors influence the timing of flowering within subspecies. This possibility is supported by results from the altitudinal experiment in Chur, where flowering phenology within subspecies was more strongly affected by elevation than by population identity (data not shown). This plasticity in flowering time would be advantageous in the heterogeneous environment of the Alps, enabling dispersed individuals to adapt plastically to their environment. To conclude, phenology is genetically differentiated between subspecies and phenology is phenotypically plastic within *C.* thyrsoides*; both phenomena are potentially adaptive.

Glacial history

Glacial history, besides adaptation to climate, is a likely candidate for allopatric differentiation between the two subspecies (Tribsch and Schönswetter 2003; Schönswetter et al. 2005). Considering the present geographical distributions of the two subspecies, differentiation during glacial survival is even likely to have widened the niche breadth of the species. A general Eastern Alpine refugium southwest of Vienna, either in the most-eastern part of the European Alps or in northwestern Slovenia (both calcareous bedrock), or in the lowland between these two regions (siliceous bedrock), has been proposed based on floristic (Merxmüller 1952, 1953, 1954) and genetic data (Schönswetter et al. 2005). Considering the present-day occurrence of *C.* carniolica*, the region of northwestern Slovenia is a potential in situ refugium where precursors of *C.* carniolica* could have survived. According to the microsatellite data (Kuss et al., unpublished results), at least three other refugia north of the Alps are candidates for the three remaining major phylogeographic groups forming *C.* thyrsoides*. Jäger (2000) explained the differentiation between the two subspecies as the result of isolated glacial survival of *C.* thyrsoides* in one or more colder refugia, to which the subspecies adapted with short, determinate flowering. It thus seems that Jäger (2000) considers *C.* carniolica* to be closer to the ancestral species and *C.* thyrsoides* as the strongly adapted subspecies, deduced from the ancestral species by survival in a high-elevation refugium. This proposition is in line with genetic data which shows that the differentiation

between the two subspecies is likely older than the differentiation within *C.* thyrsoides*, which suggests survival in climatically different refugia during the last glacial oscillation (Kuss et al., unpublished results). This glacial survival in contrasting habitats may therefore have resulted in the present allopatric distribution of the two subspecies.

Allopatric subspeciation can result from neutral processes or selection, and both usually go hand in hand (Jolivet and Bernasconi 2007; Schönswetter and Schneeweiss 2009, but see García-Verdugo et al. 2010). Traits that are strongly related to fitness are expected to adapt over time to differences in the environment. We speculate that the allopatric subspeciation of *C. thyrsoides* was initiated by glacial history, as this caused survival in separate refugia and subsequent recolonization of different regions of the Alps, whereas selection processes meanwhile caused differentiation in fitness-related traits between the respective habitats. In this study we reported genetic differentiation in several traits and we argued that some of these traits, such as flowering phenology, were likely due to adaptation to the environment. It is, however, not possible to conclude from our observations to what extent the differentiation is due to adaptation and drift. To discern between neutral genetic drift and past selection pressures on the measured traits, Q_{ST}-F_{ST} comparisons could be conducted (McKay and Latta 2002).

Conclusion

The subspecies status of *Campanula thyrsoides* subsp. *thyrsoides* and subsp. *carniolica* is corroborated by the differentiation in morphological and phenological traits observed in the common garden. From an evolutionary point of view, a quantitative description of trait variation is a precondition for an understanding of the evolutionary processes that caused the differentiation and the possible adaptive nature of the differences. The most conspicuous observation in this light is the difference in flowering behaviour between the two subspecies, *C.* thyrsoides* showing determinate and *C.* carniolica* indeterminate flowering. Determinate flowering in *C.* thyrsoides* is likely to be adaptive in the short flowering season in the high Alps, whereas indeterminate flowering may allow *C.* carniolica* to maximize fitness in the long submediterranean summers of Slovenia. This study presented an example where evolution of traits fits with the view that glacial history caused adaptive evolution through long-term survival in contrasting climates in refugia and/or during recolonisation.

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Appendix

Table 5.4: Table showing location, geographic coordinates (WGS 84) and altitude (m a.s.l.) of 24 sampled *Campanula thyrsoides* populations across the Alps and Jura Mts. Code—abbreviation as used in references (Ægisdóttir 2009; Scheepens et al. 2010; Scheepens et al., in preparation), Subsp.—thyr: subspecies *C.* thyrsoides*, carn: subspecies *C.* carniolica*, Experiment—population included in the experiment in Davos and/or Chur, *n*—sample size of individuals used in the common garden of Davos / Chur (summed over all altitudinal sites).

Location	Code	Subsp.	Experiment	Northing	Easting	Altitude	*n*
Jura, Les Amburnez	JUA	thyr	Davos / Chur	46°32'27.52"	6°13'58.57"	1340	48 / 43
Jura, Col du Marchairuz	JUM	thyr	Davos / Chur	46°33'06.27"	6°15'13.46"	1440	47 / 47
Le Chazelet	CHA	thyr	Davos	45°03'23.51"	6°16'55.49"	1757	46 / -
Col du Lautaret	LAU	thyr	Davos	45°02'03.17"	6°23'59.63"	2025	47 / -
Trient, Les Tseppes	TRI	thyr	Davos / Chur	46°02'53.93"	6°58'47.05"	2020	48 / 33
Col d'Iseran	ISE	thyr	Davos	45°23'10.42"	7°02'50.81"	2212	48 / -
Lac du Fully	FUL	thyr	Chur	46°10'09.96"	7°06'09.51"	2100	- / 30
Lac du Moiry	MOI	thyr	Davos / Chur	46°08'12.78"	7°34'02.87"	2266	48 / 21
Stockhorn	STO	thyr	Davos / Chur	46°41'37.05"	7°32'17.05"	2148	38 / 21
Schynige Platte 4	SP4	thyr	Davos / Chur	46°39'17.31"	7°54'16.67"	1911	48 / 34
Schynige Platte 6	SP6	thyr	Davos	46°39'15.23"	7°54'19.79"	1916	48 / -
Schynige Platte 18	SP18	thyr	Davos / Chur	46°39'33.73"	7°55'14.41"	1930	48 / 53
Schynige Platte 22	SP22	thyr	Davos	46°39'46.86"	7°55'57.57"	2022	44 / -
Schynige Platte 23	SP23	thyr	Davos	46°39'46.12"	7°56'14.21"	1958	49 / -
Churwalden, Joch	CHJ	thyr	Davos / Chur	46°47'51.41"	9°33'53.65"	1890	48 / 30
Langwies, Holzbüel	LAH	thyr	Davos / Chur	46°49'41.97"	9°44'00.53"	1700	42 / 33
Langwies, Listboden	LAL	thyr	Davos / Chur	46°51'07.02"	9°45'32.22"	2000	48 / 33
Ftan, Prui	FTA	thyr	Davos / Chur	46°48'32.68"	10°13'20.37"	2101	49 / 35
Sella Nevea	SEL	carn	Davos / Chur	46°23'35.00"	13°27'46.00"	932	28 / 35
Nemski Rovt	NEM	carn	Davos / Chur	46°16'23.50"	13°58'30.00"	663	49 / 44
Loibelpass	LOI	carn	Chur	46°25'51.80"	14°15'38.10"	1068	- / 19
Sklendrovice	SKL	carn	Chur	46°06'22.60"	14°59'56.10"	39	- / 13
Brodnice	BRO	carn	Davos / Chur	46°06'24.30"	15°16'53.10"	283	42 / 19
Vitanje	VIT	carn	Davos / Chur	46°22'27.80"	15°17'16.90"	422	40 / 51

Chapter 6

Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to different altitudes

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Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to different altitudes

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Abstract

Specific leaf area (SLA) is an important plant functional trait as it is an indicator of ecophysiological characteristics like relative growth rate, stress tolerance and leaf longevity. Substantial intraspecific variation in SLA is common and usually correlates with environmental conditions. For instance, SLA decreases with increasing altitude, which is understood as adjustment to temperature. It is generally assumed that intraspecific variation is mostly the result of environmentally induced phenotypic plasticity, but genetic effects may also be present, due to local adaptation or genetic drift. In this study, genotypic and environmental effects on SLA were experimentally separated for the widespread Alpine bell flower *Campanula thyrsoides* by transplanting plants to three common gardens at contrasting altitudes (600m, 1235m and 1850m a.s.l.). Seeds were sampled from 18 populations in four phylogeographic regions within the European Alps. A strong plastic response was observed: SLA decreased with increasing altitude of the common gardens (22.0% of variation). The phylogeographic regions were differentiated in SLA in the common gardens (10.1% of variation), indicating that SLA is at least partly genetically determined. Plants from the six easternmost populations experienced a submediterranean climate and showed decreased SLA values in the three common gardens compared to populations to the west, which may be explained as adaptation to drought. Within these submediterranean populations, SLA decreased with altitude of origin in two out of three common gardens. Concluding, SLA shows strong phenotypic plasticity as well as substantial genetic effects, the latter probably being the result of adaptation to local conditions rather than genetic drift.

Keywords: *Campanula thyrsoides*, phenotypic plasticity, genetic effect, local adaptation, altitude of origin

Introduction

Functional plant traits are useful for answering a broad range of research questions. At the interspecific level, functional traits can be used to explain community composition and structure (Weiher and Keddy 1999; Lavorel and Garnier 2002; Díaz et al. 2004). Functional traits can also be used to classify plant species according to strategies sensu Grime (1977) or to explain the occurrence and distribution of species (Díaz et al. 1998). At the intraspecific level, func-

tional traits often show considerable variability which is usually assumed to be of adaptive significance.

One of the most informative functional traits is specific leaf area (SLA) (Lavorel and Garnier 2002; Wright et al. 2004; Westoby and Wright 2006; Poorter et al. 2009) as it is an indicator of ecophysiological characteristics such as relative growth rate, stress tolerance and leaf longevity (Weiher et al. 1999; Wright and Westoby 2002). It is calculated as one-sided leaf surface area per unit dry weight. A higher SLA indicates decreased leaf thickness, decreased leaf density, or a combination of both. Poorter et al. (2009) report SLA to range interspecifically from 5 cm^2g^{-1} to >3333 cm^2g^{-1}, but within-species variation can be large as well. For example, Cordell et al. (1998) report SLA for the Hawaiian endemic Metrosideros polymorpha to range from 24.6 cm^2g^{-1} to 93.5 cm^2g^{-1} across an altitudinal gradient; an almost 4-fold difference. SLA correlates strongly with temperature, irradiance and water availability (Poorter et al. 2009). Among as well as within species, SLA generally decreases with increasing altitude (Morecroft and Woodward 1996; Körner 2003, p. 212; Milla et al. 2009). This is most likely a response to decreasing temperature rather than increasing radiation (Körner 2003) or changes in water availability (Poorter et al. 2009).

It is generally assumed that functional traits are highly plastic. Yet whether and to what extent intraspecific variation in functional traits is due to strict genetic control, due to environmentally induced phenotypic plasticity or a combination of both is an open question. There have been reports of genetic effects on SLA (e.g. Morecroft and Woodward 1996). However, most studies focus on phenotypic plasticity alone (Poorter et al. 2009). Phenotypic plasticity allows a genotype to adjust its trait values to a range of environmental conditions (Bradshaw 1965; Schlichting 1986; Sultan 2000). For instance, in the case of SLA, values may change as new leaves develop, conferring continuous adaptation to the environment during plant growth (Sims and Pearcy 1992). In contrast, any genetic effects influencing SLA may hamper such rapid adjustment; in that case, changes in SLA come about by natural selection of those individuals possessing trait values that are well adapted to the current environment - a process taking place on a time scale of generations (Jump and Peñuelas 2005). Thus, phenotypic plasticity allows for a more rapid and flexible adjustment to environmental change as compared to genetically fixed adaptations (Sultan 2000). It follows that the extent to which SLA is phenotypically plastic or genetically fixed has important implications for the survival probability of populations under changing environmental conditions.

By growing plants from different populations in a common environment, common garden experiments can reveal genetic differentiation in traits among those populations (Turesson 1922, Clausen et al. 1940). When using multiple common gardens situated in contrasting environments, phenotypic plasticity can be shown by different trait values among the gardens. To prove local adaptation, common garden experiments do not suffice and reciprocal transplantation experiments are necessary (Kawecki and Ebert 2004). However, the case for local adaptation can be strengthened by other sources of evidence. For instance, local adaptation is likely if a trait measured in a common garden correlates with an environmental variable at the locations of origin (Linhart and Grant 1996; Ennos 2001). In addition, the case for local adaptation is strengthened if drift can be excluded by showing absence of isolation-by-distance and by demonstrating a significantly stronger or weaker quantitative differentiation in the studied traits compared to neutral molecular differentiation (Q_{ST} vs. F_{ST} comparisons, Rogers 1986; Spitze 1993; McKay and Latta 2002).

In this study, genotypic and environmental variation in specific leaf area are experimentally separated for *Campanula thyrsoides* L. (Campanulaceae), a monocarpic species widespread in the European Alps. Seeds were collected from 18 populations from four major Alpine biogeographic regions and across a large altitudinal range, and derived plants were grown in three common gardens at contrasting altitudes. The biogeographic regions probably reflect Quaternary postglacial recolonization patterns (Merxmüller 1952, 1953, 1954; Ozenda 1988). Intraspecific differentiation of neutral genetic markers of several plant species was shown to correlate with these four biogeographic re-

gions (Schönswetter et al. 2005), and is likewise described for *C. thyrsoides* (Kuss et al., unpublished). After ten weeks growth in the common gardens, a response of SLA to the altitude treatment would indicate phenotypic plasticity, whereas differentiation among regions or populations would indicate genetic effects (Bradshaw 1965, 1984; Galen et al. 1991; Sultan 2000). Significant interactions between the altitude treatment and regions or populations would indicate genetic variation in phenotypic plasticity. In order to investigate whether any genetic effects were due to local adaptation or genetic drift (Linhart and Grant 1996), SLA was correlated with altitude of population origin, while accounting for climatic differences between the Eastern Alps and the higher populations to the west. Altitude of origin functioned as a substitute for a score of environmental variables changing with altitude, most notably temperature (Monty and Mahy 2009). Additionally, isolation by distance in SLA was determined and a Q_{ST} vs. F_{ST} comparison was performed to check for past natural selection on this trait.
In summary, this report investigates the following three research questions: 1) Does the functional trait of SLA show phenotypic plasticity in *Campanula thyrsoides*? Specifically: does SLA respond to transplantation to different altitudes? 2) Are there any genetic effects at the regional or population level? 3) Is there any evidence suggesting local adaptation of SLA?

Methods

Campanula thyrsoides L. is a monocarpic bell flower occurring in subalpine and alpine calcareous grasslands. Its main distribution is across the Jura and the European Alps. The altitudinal range is typically from 1600 to 2200m a.s.l. (Kuss et al. 2007), although it can reach much higher altitudes (2900m a.s.l., Val Mora, Switzerland, Brunies 1906, in: Kuss et al. 2007) as well as much lower altitudes, especially in the Dinaric Alps, where the lowest population has been found at 217m a.s.l. (Gracnica, Slovenia, J. Stöcklin, pers. obs.). The species occurs in isolated populations, yet can be locally abundant. Initiation of flowering is dependent on the rosette size, and flowering age ranges from 3 to 16 years with an average of about 10 years (Kuss et al. 2007, Kuss et al. 2008).
Campanula thyrsoides is taken as a model for this study, because the species rapidly replaced its leaves after transplantation to different altitudes, which allowed investigation of changes in SLA within a short time frame. *C. thyrsoides* seeds were sampled from several mother plants from 18 populations over a broad altitudinal range (283–2266m a.s.l.) across four biogeographic regions spanning the Jura and the Alps (Ozenda 1988; Table 6.1). The biogeographic regions are denoted from west to east as Western Alps, Central Swiss Alps, Central Austrian Alps and Eastern Alps. Starting in September 2007, seeds were germinated on moist filter paper in Petri dishes while kept under controlled light and moisture conditions in a greenhouse located in Basel, Switzerland (276 m a.s.l.). After germination, seedlings were planted into 4cm diameter pots filled with low-nutrient soil (Anzuchterde, Ökohum, Herrenhof, Switzerland). After 10-18 weeks, plants were repotted into pots of 10cm × 10cm × 10cm with potting soil (Topferde Ökohum, Herrenhof, Switzerland). Insecticide was sprayed several times during growth to control Aphidoidea and Sciaridae outbreaks. Fertiliser was added once during growth in the greenhouse. In spring, plants were transferred to an outside garden to acclimatise before final transplantation.
On 24-Jun-2008, rosette diameter of each plant was measured to the nearest 0.5 cm and the plants were transplanted to common gardens in three fenced sites at different altitudes (600m, 1235m and 1850m a.s.l.) on the southeast-facing slope of Mt. Calanda, near Chur, Switzerland. Plants were planted into the local soil which was topped with sterilized soil to limit weed germination. The distances between the three sites did not exceed 2500m based on the longitudinal and latitudinal positions. It should be noted that immediately after transplantation, the plants were observed to replace their leaves. On 2-Sep-2008 (ten weeks after transplantation), rosette diameter of each plant was measured again, and a total of 594 plants were sampled for SLA: 204 individuals at the lowest site (600m), 183 at the intermediate site (1235m) and 207 at the highest site (1850m). Each altitudinal site contained on average 49.5 (range: 34-64) plants per region and 11 (4-19) plants

per population. For the SLA measurements, five circular leaf corings were taken from newly grown, mature leaves. The diameter of corings was either 5.5mm or 9mm depending on the size of the leaf. The main leaf vein was avoided during coring. Leaf corings were stored in small parchment bags and were transported to the laboratory within 24 hours, dried for 48 hours at 60 °C and subsequently stored in a desiccator with silica gel. All leaf corings of one individual were weighed together to a precision of 0.0001g. The average weight of five corings of 5.5 and 9mm diameter was 0.0079g (s.d. 0.0022g) and 0.0220g (s.d. 0.0041g), respectively. SLA was calculated as the total one-sided fresh leaf area divided by the dry weight. On 24-May-2009, rosette diameter was measured again and leaf thickness of one leaf per plant was measured on all transplanted plants to a precision of 0.01mm using a Teclock SM-112 dial thickness gauge (Okaya, Japan).

Table 6.1: Locations of sampled *Campanula thyrsoides* populations and the experimental sites indicating region, latitude, longitude, altitude (m a.s.l.), annual precipitation and yearly minimum, mean and maximum temperatures (based on monthly averages; Hijmans et al. 2005). WA, Western Alps, CSA, Central Swiss Alps, CAA, Central Austrian Alps, EA, Eastern Alps

Sampling site	Code	Region	Northing	Easting	Alt	Prec	T_{min}	T_{mean}	T_{max}
Jura, Les Amburnez	JUA	WA	46°32'27.52"	6°13'58.57"	1340	1539	-6.5	5.1	18.8
Jura, Col du Marchairuz	JUM	WA	46°33'06.27"	6°15'13.46"	1440	1695	-7.5	3.9	17.2
Trient, Les Tseppes	TRI	WA	46°02'53.93"	6°58'47.05"	2020	1672	-9.3	1.9	14.8
Lac du Fully	FUL	WA	46°10'09.96"	7°06'09.51"	2100	1780	-9.8	1.0	13.3
Lac du Moiry	MOI	WA	46°08'12.78"	7°34'02.87"	2266	1827	-10.3	-0.1	11.6
Stockhorn	STO	CSA	46°41'37.05"	7°32'17.05"	2148	1700	-8.3	2.3	14.4
Schynige Platte, pop 4	SP4	CSA	46°39'17.31"	7°54'16.67"	1911	1716	-8.5	2.0	13.8
Schynige Platte, pop 18	SP18	CSA	46°39'33.73"	7°55'14.41"	1930	1716	-8.5	2.0	13.8
Churwalden, Joch	CHJ	CAA	46°47'51.41"	9°33'53.65"	1890	1520	-9.6	0.2	10.8
Langwies, Holzbüel	LAH	CAA	46°49'41.97"	9°44'00.53"	1700	1095	-7.7	3.6	16.1
Langwies, Listboden	LAL	CAA	46°51'07.02"	9°45'32.22"	2000	1326	-9.1	1.1	12.3
Ftan, Prui	FTA	CAA	46°48'32.68"	10°13'20.37"	2101	1383	-10.6	-1.1	9.3
Sella Nevea	SEL	EA	46°23'35.00"	13°27'46.00"	932	1253	-8.7	4.0	18.1
Nemski Rovt	NEM	EA	46°16'23.50"	13°58'30.00"	663	1242	-7.7	6.0	21.4
Loibelpass	LOI	EA	46°25'51.80"	14°15'38.10"	1068	1219	-5.0	8.8	24.8
Sklendrovice	SKL	EA	46°06'22.60"	14°59'56.10"	39	1180	-6.6	7.3	23.1
Brodnice	BRO	EA	46°06'24.30"	15°16'53.10"	283	1132	-5.6	9.5	25.6
Vitanje	VIT	EA	46°22'27.80"	15°17'16.90"	422	1086	-5.6	8.9	25.0
Experimental site			Northing	Easting	Alt				
Lowest site			46°52'18.34"	9°31'05.45"	600				
Intermediate site			46°53'11.29"	9°29'42.76"	1235				
Highest site			46°52'48.69"	9°30'36.10"	1850				

Data analysis

To separate genetic and environmental effects on SLA, a hierarchical mixed-model ANCOVA using type III sum of squares was performed, employing a restricted maximum-likelihood approach, which is robust to unbalanced sampling designs. The following factors were tested: rosette diameter (fixed covariable), altitude treatment (fixed factor), region (fixed factor), population (random factor, nested in region) and the two-way interactions of altitude treatment with region and population. Rosette diameter at the time of transplantation was incorporated as a fixed covariable in order to remove a possible effect of plant age on SLA. The type III sums of squares were calculated using JMP (SAS Institute Inc. 2003, Version 5.0.1.2), whereas degrees of freedom, mean squares, F-ratio's and P-values were calculated by hand. Tukey HSD post-hoc tests (α=0.05) were performed in JMP to locate any significant differences for altitude treatment and region. Variance components were calculated by hand using weighted number of replicates per level.

All sampling sites in the Eastern Alps region are located at lower elevations compared to the regions to the west (Eastern Alps: 283-1068m a.s.l.; Western Alps, Central Swiss Alps and Central Austrian Alps: 1340-2266m a.s.l., Table 6.1), which probably affects climate. Climatic data for each sampled population were obtained from WorldClim (www.worldclim.org) which offers interpolated climate surfaces on a 2.5 arc-minutes scale based on measurements from 1950-2000 (Hijmans et al. 2005). From the four possible WorldClim datapoints surrounding a population location, the one with the least altitudinal difference to the population location was chosen. Temperature data for any altitudinal difference was corrected for by subtracting or adding 0.0055 °C per meter increase or decrease in altitude, respectively (Ozenda 1988). Using yearly precipitation and yearly minimum, mean and maximum temperature (based on monthly averages), the climatic data indicates that populations in the Eastern Alps region experience a submediterranean climate. This is characterized by higher temperatures and lower precipitation compared to the populations in the other three regions (Table 6.1). K-means clustering analysis of a dataset including climatic data and altitude of origin also showed that the Eastern Alps were clearly separated from the other three regions (data not shown). To account for these differences, Pearson correlations were performed between population-level SLA and altitude of origin on populations of the Eastern Alps and of the other regions separately. Coefficient of variation of population-level SLA across the three altitude sites was also correlated with altitude of origin for these two groups.

A Mantel test (Mantel 1967) was performed on pairwise population-level differences in SLA in relation with pairwise geographic population distances using 10'000 permutations to investigate isolation by distance. The k-means clustering analysis, the correlation analyses and the Mantel test described above were performed using the R statistical package (www.r-project.org, R Development Core Team 2009; version 2.9.2 using packages "cluster" and "ade4").

To investigate the presence of natural selection on SLA, quantitative trait differentiation in SLA (Q_{ST}) was compared with the neutral molecular differentiation index G'_{ST}. The latter was calculated using previously obtained microsatellite data of the populations involved (Ægisdóttir et al. 2009, Kuss et al. unpublished). As a first step, narrow-sense heritabilities (h^2) of SLA were calculated for each population to investigate the presence of additive genetic differences among individuals (Petit et al. 2005). A reduced dataset was used containing at least two replicates per seed family per altitude and at least two seed families per altitude. Due to this restriction, six out of 18 populations had to be excluded from the dataset because of lacking replicates (BRO, CHJ, JUA, LAH, LAL, MOI; Table 6.1). Narrow-sense heritabilities were calculated as $h^2 = 4V_F/(4V_F + V_E)$ where V_F is the seed family variance and V_E is the residual variance. These two variance components were obtained from type III SS random models which included rosette diameter, altitude treatment and seed family. Q_{ST} of SLA was estimated among all populations based on the same dataset. Using a half-sib design, Q_{ST} equals $V_P/(8\times V_F+V_P)$, where V_P is the population variance. A similar model was used to obtain the variance components V_P and V_F, except that population was added as a random factor and seed familiy became nested in population. In both models for h^2 and Q_{ST}, region was left out of the analysis because this would remove genetic variability which should be attributed solely to the population and seed family levels in order to properly calculate V_P and V_F. Interactions of altitude treatment with population and seed family were omitted due to insufficient replicates. Additional Q_{ST} values were calculated for the five populations from the Eastern Alps and for the seven populations from the other three regions separately. All models were performed using the R statistical package (R Development Core Team 2009; version 2.9.2 using package "lme4"). Based on the same restricted set of populations, the neutral genetic differentiation index G'_{ST} (Hedrick 2005) was calculated among all populations and among the Eastern Alps and the other regions separately using SMOGD 1.2.5 (Crawford 2009). The two populations from Schynige Platte (SP18 and SP4) were not genotyped in the mentioned studies. Instead genetic data from two other populations nearby were used (SPO and SPU in Ægisdóttir et al.

2009). Variances of both indices were obtained by using the Jackknife procedure over populations, and the difference between Q_{ST} and Q'_{ST} was tested with a t-test.

Results

Phenotypic plasticity and genetic effects

Specific leaf area decreased with increasing altitude of transplantation site (Fig. 6.1; $P<0.0001$, Table 6.2) and altitude treatment explained 22.0% of the variance in the data (Table 6.2). Mean SLA values of individuals from the low, intermediate and high sites were 164.9 ± 2.2 (mean ± s.e.), 161.6 ± 2.0 and 132.4 ± 1.3 cm^2g^{-1}, respectively. A Tukey HSD post-hoc test on altitude treatment revealed that the highest site was significantly different from the low and intermediate site, whereas the two lower sites did not significantly differ from each other (Fig. 6.1). This amounts to a reduction of 18.1% in SLA across 615m altitudinal difference between the intermediate and highest site. However, compared with SLA values of a set of untransplanted plants which had the remarkably higher average SLA of 268.5 cm^2g^{-1}, all transplanted individuals had strongly decreased SLA values (One-way ANCOVA with rosette diameter as covariable, $F_{1,681}$=950.8, $P<0.0001$, data not shown).

Region was highly significant ($P<0.0001$, Table 6.2) and accounted for 10.1% of the variation. A Tukey HSD post-hoc test on region showed that the Western Alps, Central Swiss Alps and Central Austrian Alps were significantly different from the Eastern Alps. Population and the interactions of altitude treatment with region and population were not significant (Table 6.2) and explained only small amounts of the variance. Unexplained variance amounted to 63.8%. The highly variable rosette diameter (range 3-36.5; mean (s.e.) = 14.16 (0.26)) covaried significantly with SLA but explained only 2.1% of the variance (Table 6.2). Within altitudinal sites leaf thickness decreased with increasing SLA (Pearson correlations, 600m: r_{146}=-0.23, P=0.0046; 1235m: r_{69}=-0.43, P=0.00016; 1850m: r_{101}=-0.42, $P<0.0001$). Across altitudinal sites, leaf thickness decreased (mean ± s.e.; 600m: 0.36 ± 0.01mm; 1235m: 0.31 ± 0.01mm; 1850m: 0.30 ± 0.00mm; F_2,585=58.9, $P<0.0001$) between the low and the intermediate site (Tukey HSD post-hoc test, P=0.05).

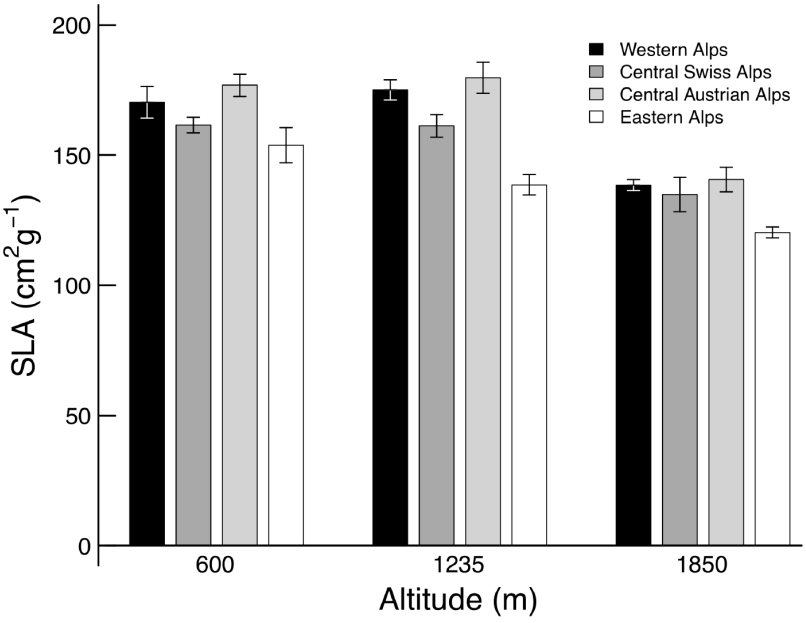

Figure 6.1: Specific leaf area (SLA) of *Campanula thyrsoides* leaves from four biogegraphic regions, measured ten weeks after transplantation to three altitudes. Values are population means ± SE

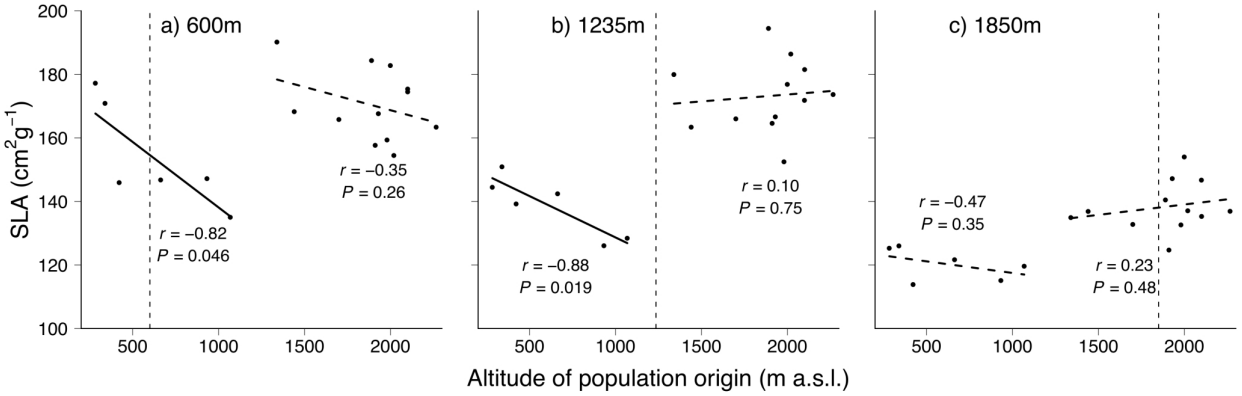

Figure 6.2: Correlations between SLA and altitude of population origin at each of the three experimental altitudinal sites: a) 600m, b) 1235m and c) 1850m a.s.l. Data points represent mean population SLA for each altitudinal site. Pearson's correlation coefficient r and P-values are given for each correlation. Separate correlations were calculated for populations from the Eastern Alps (left) and the other regions (right). Solid and dashed regression lines indicate significant and non-significant correlations, respectively

Table 6.2: Summary of results from ANCOVA of environmental (three common gardens) and genetic effects (region and population) on specific leaf area. Population is treated as a random factor and is nested in region. % proportion of variance component

	df	SS	MS	F	P	V	%
Rosette diameter	1	10222	10222	20.57	<0.0001	16.37	2.10
Altitude treatment	2	68594	34297	69.01	<0.0001	170.96	21.97
Region	3	36545	12182	19.29	<0.0001	78.87	10.14
Population (Region)	14	8839	631	1.27	0.2214	4.10	0.53
Alt treatment × Region	6	6195	1033	2.08	0.0542	10.86	1.40
Alt treatment × Population (Region)	28	5229	187	0.38	0.9986	0.00	0.00
Residuals	539	267879	497			496.99	63.87
Total	593	403504				778.15	100.00

Adaptation vs. genetic drift

Pearson correlations of population means of SLA with altitude of origin yielded significant negative relationships only for the populations from the Eastern Alps at the low and intermediate site (r=-0.82, P=0.046 and r=-0.88, P=0.019 respectively, Fig. 6.2a and 6.2b). The coefficient of variation across altitudinal sites showed a negative relationship with altitude of origin for the Eastern Alps (r=-0.83, P=0.041) but not for the other three regions (Fig. 6.3).
The Mantel test indicated that pairwise population differences in SLA did not correlate with pairwise geographic distances (r=-0.047, P=0.74). The range in narrow-sense heritabilities in the twelve investigated populations was large (0.00-0.72; mean ± s.e. 0.17 ± 0.07) and the mean differed significantly from zero (one-sample t-test: t_{11}=2.3, P=0.042). The among-population Q_{ST} was 0.53 (CI 0.47-0.59) whereas G'_{ST} was 0.73 (CI 0.70-0.77), and 95% confidence intervals of Q_{ST} did not overlap with those of G'_{ST} indicating stabilizing selection. Q_{ST} was 0.22 (CI -0.17-0.61) and 0.23 (CI 0.02-0.44) for the Eastern Alps and the three regions respectively, which were both lower than their respective G'_{ST} values of 0.65 (CI 0.58-0.73) and 0.55 (CI 0.48-0.62), indicating comparatively strong stabilizing selection within these regions. However, the confidence intervals of the Eastern Alps' Q_{ST} and G'_{ST} values overlapped, likely due to small sample size.

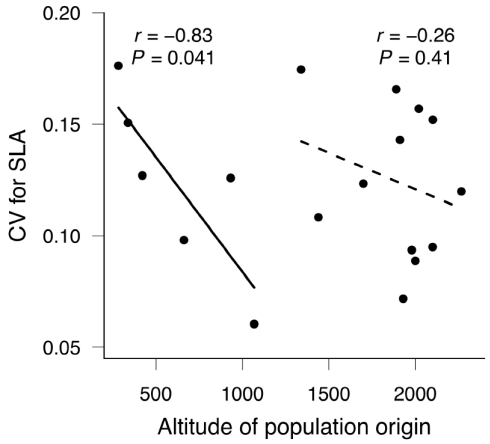

Figure 6.3: Coefficient of variation of population-level SLA among the altitudinal sites (i.e. the experimental sites at 600m, 1235m and 1850m a.s.l.) as a function of altitude of population origin; n=3 for each population. Pearson's correlation coefficient r and P-value were determined separately for Eastern Alpine populations (left) and populations from the other three regions (right)

Discussion

Phenotypic plasticity

SLA showed a substantial phenotypic plasticity as indicated by the significant decrease in SLA with increasing altitude across the altitude sites ten weeks after transplantation (Table 6.1, Fig. 6.1). The Tukey HSD post-hoc tests showed that this was due to lower SLA values at the highest site compared to the low and intermediate sites. This indicates that the low and intermediate sites exerted equal pressure on the plants after transplantation, resulting in similar SLA values, whereas the highest site exerted stronger pressure on the plants. This effect could be caused by similar environmental conditions at the low and intermediate sites or by the exceeding of an environmental threshold value (e.g. in temperature) between the intermediate and high site to which SLA reacts sharply.
All SLA values decreased in comparison to plants which remained in the greenhouse. The higher SLA values for these control plants were likely to be due to other factors besides altitude, such as decreased light influx in the greenhouse (Poorter et al. 2009) and increased shading due to the higher density positioning of the plants. These data can therefore not be quantitatively compared to the SLA of the transplanted plants. However, clear is that all plants showed a reaction to the transplantation. This allows for the conclusion that SLA is highly phenotypically plastic and is able to react immediately and rapidly to changing environmental conditions such as those at contrasting altitudes.
The decrease in SLA with increasing altitude can be realized through increasing leaf density and increasing leaf thickness (Körner 2003; Atkin et al. 2005; Poorter et al. 2009). In deciduous herbs, both increased density and thickness are often observed; the former through limited cell expansion leading to smaller cells and more cells per unit leaf volume, the latter through increase in the number of palisade parenchyma layers (Körner 2003; Poorter et al. 2009). These changes are explained as an investment in photosynthetically active tissue to promote efficiency in light capture (Körner 2003), and according to Woodward (1983), this behaviour optimizes competition ability in colder climates. Leaf thickness measurements from 24-May-2009 unexpectedly showed that mean leaf thickness decreased with increasing altitude. Using the relationship SLA^{-1} = leaf density × leaf thickness, it can be deduced that leaf density must have increased substantially with altitude to yield the observed SLA values; this may be due to limited cell expansion.

Genetic effects

The effect of region was significant and amounted to nearly half the variance due to plasticity (Table 6.2) indicating that SLA is partly constrained by genetic effects. This region effect is in line with phylogeographic studies

that report a genetic patterning across biogeographic regions (e.g. Schönswetter et al. 2005) which has been explained as a result of genetic drift. The present results indicated that only the Eastern Alps were genetically differentiated from the other regions. This is in accord with microsatellite data showing that neutral genetic differentiation was highest between the Eastern Alps versus the other three regions (AMOVA of Eastern Alps vs. other three regions: 7.8%; Kuss et al., unpublished). Conversely, a cluster analysis separated these two groups based on climate and altitude of origin (see methods). Therefore, this regional differentiation is more likely to be due to local adaptation to the submediterranean climate in the Eastern Alps and to the alpine conditions in the other three regions rather than to genetic drift.

Maternal effects offer an alternative explanation to the region effect. However, maternal effects have been shown to be most prominent in the seedling phase and diminish over time (Roach and Wulff 1987). Thus, given the age of the plants (all plants were circa nine months old at the time of transplantation), it is likely that maternal effects can be rejected as explanation of the results.

Populations are expected to differ in trait values observed in common garden experiments, since naturally populations occur in different environments to which they are likely to be locally adapted (Jump and Peñuelas 2005). In the present study, the population factor was not significant, indicating genetic differentiation was absent among populations within regions. This finding suggests that either environmental conditions are similar among the populations removing the need for local adaptations, or, more likely, that phenotypic plasticity is strong enough to adjust SLA to a broad range of environmental conditions. The latter can be an evolutionary answer to strong temporal variation as well as to strong spatial variation in environmental conditions (Pigliucci et al. 2003), experienced by the species through pollen and seed dispersal in the heterogeneous Alpine landscape.

Rosette diameter at the time of transplantation correlated significantly with SLA in the model, which implies that age differences could have influenced SLA. The inclusion of rosette diameter in the model helped to correct for this experimental error. However, since plants from the Eastern Alps are generally larger in diameter than plants from the other regions (mean ± s.e. in cm: 19.4 ± 0.52 and 14.2 ± 0.26, respectively, data not shown), rosette diameter could reduce explanatory power from the region factor. Nevertheless, rosette diameter accounted only for a small part of the variance compared to the region effect, and region remained highly significant. The interactions of altitude treatment with region and population were not significant, which indicates that plasticity in SLA at the region and population levels were always in the same direction across the altitudinal gardens, i.e. a decrease in SLA with increasing altitude.

The variance components (Table 6.2) show substantial environmental as well as genotypic effects on SLA (22.0% vs. a sum of 12.0%). However, this result is due in part to the practicalities of the experiment; an even larger range in altitudinal sites would likely also have increased the environmental effect, considering that SLA reached much higher values in the greenhouse.

The question can be raised whether ten weeks is enough time for the newly formed leaves to reach stable SLA levels. In deciduous tree species, SLA starts off low after bud burst, increases during leaf expansion and then decreases again to stable levels within 30 days (Jurik 1986). A similar pattern has been observed in wheat (Rawson et al. 1987). Comparing the average rosette diameter of the highest site at the date of sampling (2-Sep-2008) with the subsequent year (24-May-2009) did not result in a significant difference (one-sided t-test with unequal variances, t_{386}=1.06, P=0.15, data not shown). This suggests that at the time of sampling, leaves of plants at the highest site, although small in size, were fully mature and not delayed in their development as compared to the low and intermediate sites. These observations suggest that time between transplantation and measurement is long enough for SLA to reach stable levels.

Adaptation vs. genetic drift

Any differentiation in SLA by region and population (Table 6.2) could theoretically be either the result of selection leading to adaptation or the result of neutral genetic drift (Linhart and

Grant 1996; Waldmann and Andersson 1998; Petit et al. 2001). The observed differentiation between the Eastern Alps and the other regions is most likely the result of adaptation to opposing climates rather than to drift. The Eastern Alpine populations are subject to a submediterranean climate, with a higher probability of summer droughts. In fact, drought (due to dry summers as well as to high mean temperatures increasing evapotranspiration) has been found to correlate with lower SLA interspecifically (Niinemets 2001) as well as intraspecifically (Fernández et al. 2002; Poorter et al. 2009). This can also be observed from Figure 6.2 where the six Eastern Alpine populations to the left of each graph show decreased SLA values as compared to the other populations. The heavier leaves (i.e. lower SLA) in the Eastern Alpine populations could be an adaptation to local climatic conditions, possibly through more densely cutinized epidermal walls and a thicker wax layer (Larcher 2003, p. 410; Galmés et al. 2005).

To investigate the presence of genetic adaptation to altitude, correlations were performed of SLA against altitude of population origin for the two groups of populations separately (Eastern Alps and the other regions). Adaptation to altitude of origin may be expected based on the well-documented negative relationship between SLA and altitude (Cordell et al. 1998; Körner 2003). The significantly decreasing SLA with altitude of origin for the Eastern Alpine populations at the low and intermediate sites corroborate this pattern. Since the population factor was not significant in the ANCOVA, absence of any differentiation in SLA is the best explanation for the absence of correlations in the other three regions.

The coefficient of variation of SLA (Fig. 6.3) showed a significant negative relationship for the Eastern Alps, indicating that the populations from lower altitudes exhibited a larger range in plasticity than those from higher altitudes within the Eastern Alps. Whether this is because the higher-altitude populations were not able to respond as strongly as the lower-altitude populations or because they did not need to adjust SLA values at the three altitudinal sites, remains an open question.

The non-significant result of the Mantel test indicates that isolation by distance is not a good predictor of SLA. Hence, drift cannot explain the geographic distribution of populations' mean SLA values. The significant heritability of SLA indicated evolutionary potential and the Q_{ST} vs. G'_{ST} comparison showed that Q_{ST} was high but significantly lower than G'_{ST}, indicating stabilizing selection on SLA. Since the population factor was not significant in the model, the high Q_{ST} of 0.53 should not be understood as evidence for strong population differentiation, but rather as strong regional differentiation between the Eastern Alps and the other regions. Indeed, analysis of the populations from the Eastern Alps and the other regions separately resulted in substantially reduced Q_{ST} values of 0.22 and 0.23 respectively. Since these were much lower than the respective G'_{ST} values, this is an indication for strong stabilizing selection. It can be concluded that stabilizing selection acted as a weak conservative force in the strongly divergent evolution of the Eastern Alps versus the other three regions, and as a strong conservative force within the regions.

Strong plastic response of transplanted plants to their respective altitudes are well-known from literature and has been interpreted as adjustment to temperature (Körner 2003). The present results suggest that populations of *C. thyrsoides* would be able to survive under current climate change by adjusting their SLA plastically. This adjustment by means of phenotypic plasticity may allow populations in the long run to adapt genetically to the environmental changes via genetic assimilation (Price et al. 2003).

Conclusions

Phenotypic plasticity in SLA is a rapid and strong process in *Campanula thyrsoides*, but a substantial genetic component of SLA is also present. This genetic component was shown to be due to genetic adaptation rather than to drift, and appeared at two levels. First, populations in the Eastern Alps showed lower SLA values compared to populations from the other regions, which is likely to be an adaptation to the submediterranean climate. Second, within the Eastern Alps, populations showed decreasing SLA and decreasing variation in SLA with increasing altitude. Additionally, the absence of

isolation by distance suggests that genetic drift cannot explain the geographic pattern observed for SLA. Finally, the Q_{ST} vs. G'_{ST} comparison indicated that stabilizing selection for SLA was stronger within the regions than between them, reflecting the adaptive differentiation in SLA between the Eastern Alps and the other regions.

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Chapter 7

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

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

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Abstract

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. 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 bow. 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. 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.

Keywords: *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 land-use 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 and 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). On the larger scale at 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 and 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 and 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 and Kudo, 2004; Pluess and Stöcklin, 2004). Genetic bottlenecks are likely, because only a few individuals may have colonized unoccupied habitats and, in addition, genetic exchange among established habitats is restricted (Wade and McCauley, 1988). Moreover, as a consequence of such small population sizes, inbreeding due to a loss of genetic diversity might occur (Ellstrand and 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 and Templeton, 1999), which has been found frequently in the Alps (Pluess and 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 and 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. 7.1A). 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. 7.1B). 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 analyze 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 bow (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 whether 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?

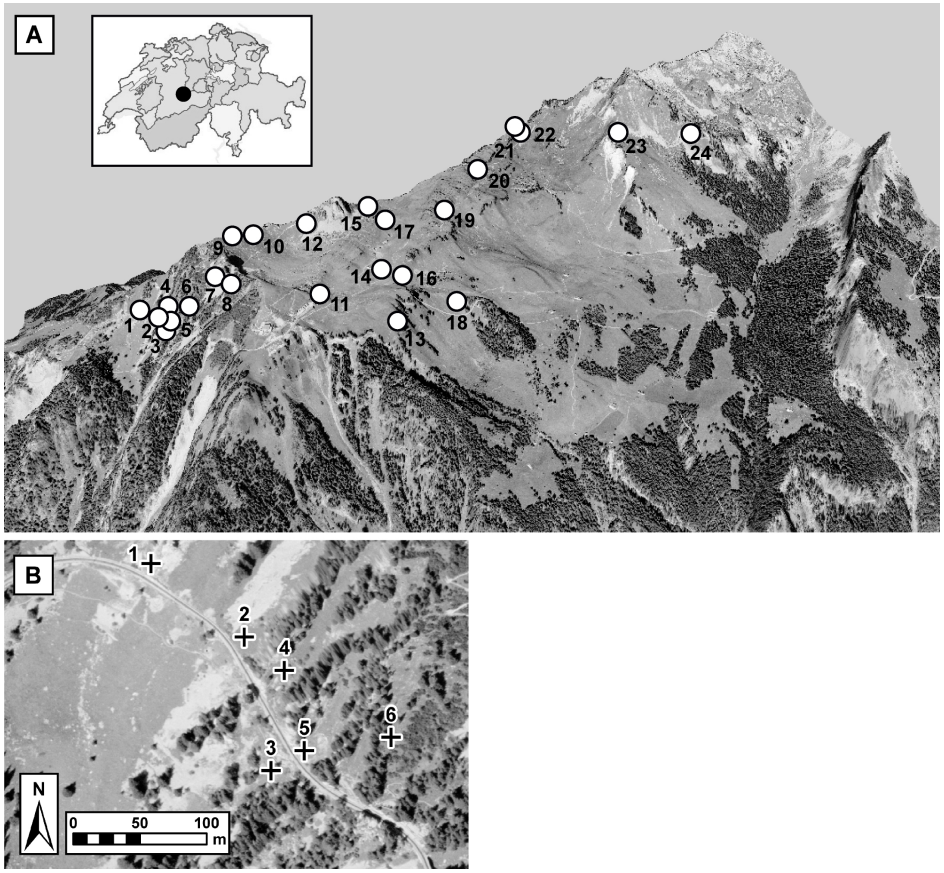

Figure 7.1: Study region on the mountain plateau of the Schynige Platte located in the Swiss Alps with (A) all studied 24 populations of *Campanula* thyrsoides and (B) a cut-out of the study region with six populations of close proximity. For geographical distances among populations see Appendix Table 7.7. Reproduced by the permission of Swisstopo, Berne, Switzerland (BA100596).

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 1000–2900m 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 et 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 1800–2100 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, accessable and spatially separated populations of *C. thyrsoides* (Fig. 7.1). 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 7.1). The number of flowering individuals was counted during peak flowering in each summer of the subsequent four years.

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; Selkoe and Toonen, 2006) as neutral molecular markers to reach high enough resolution for detection of the smallest genetic differences among individuals in such a small region (Luikart and England, 1999; 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: Campthy1, Campthy3, Campthy5, Campthy6, Campthy9 (for details see Appendix Table 7.6).

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%.

Table 7.1: 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.[a]	Slope (°)	Area[b] (m^2)	Vegetation cover[c] (%)	Density[d] (per m^2)	Size[e]	No. flowering[f]
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 (1-9)
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 7.4). *Site lies within the Botanical Garden. [a]Exposition; [b]Area occupied by a population; [c]Coverage of herb layer; [d]Plant density; [e]Population size (number of flowering and non-flowering individuals); [f]Number of flowering individuals.

Genetic differentiation

To estimate genetic differentiation among all populations, we calculated Wright's F_{ST} (Weir and Cockerham, 1984) and 95% confidence intervals using the program GENETICS version 4.05 (Belkhir et al., 2004). G_{ST} (Nei, 1973) and 95% confidence intervals were obtained with 1000 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 and 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 cluster analyses. For the assignment of individuals to genetic clusters, we used the program STRUCTURE version 2.3 (Hubisz et al., 2009). We selected the "no 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 5000 random permutations. The pairwise genetic differences (F_{ST}) were calculated using the same program and were tested with 1000 permutations at a significance level of $P = 0.05$. 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 1000 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 and 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 and 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 and Thompson, 1992) using 10 000 permutations, 20 batches, 5000 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 7.1). 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 obtained by 1000 permutations. Both mutation models are assumed for microsatellite evolution, but the TPM might be more appropriate for most microsatellites (Goldstein and Pollock, 1997; Balloux and Lugon-Moulin, 2002).
In populations that experienced a genetic bottleneck during colonization, 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 and 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 loci pairs.

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 (ΔK; see Appendix Figure 7.5) for results of clustering analysis with STRUCTURE revealed two clusters of uneven size to capture the genetic structure best (Fig. 7.2). For the most likely partion, the spatial clustering analysis using BAPS also revealed two groups, including the same populations (Fig. 7.3).
The variance components inferred from the AMOVA were all significant with F_{ST} = 0.076 for differentiation among the two spatial groups and F_{SC} = 0.052 for differentiation among populations within groups (Table 7.2). F_{ST} of pairwise genetic differentiation ranged from 0.001–0.191 (see Appendix Table 7.7), whereby 232 out of all 276 comparisons were significant. The pairwise geographic distances ranged from 11–2710 m with a mean (± SD) of 999 m (± 675 m) (see Appendix Table 7.7). 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. 7.4).

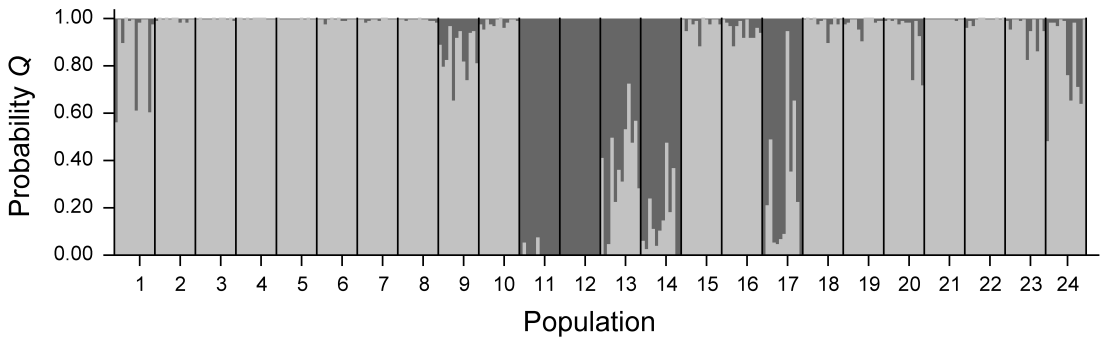

Figure 7.2: Results of a cluster analysis with the program STRUCTURE (Hubisz et al., 2009) using microsatellite data of 24 populations of *Campanula thyrsoides* from the Schynige Platte for the most likely number of clusters $K = 2$. The different clusters are represented by different shades of grey. 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.

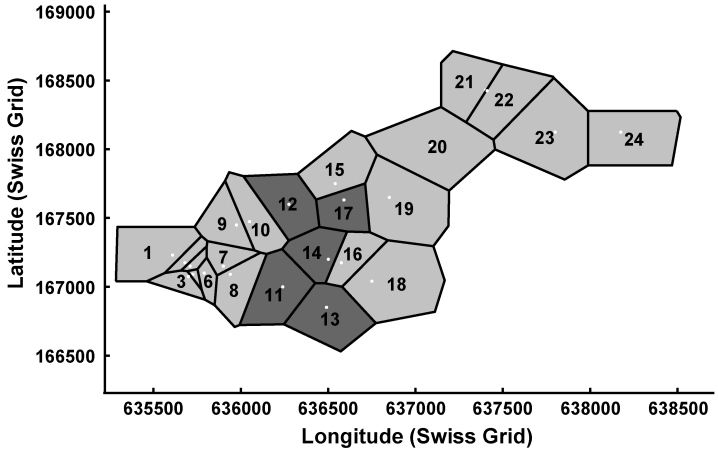

Figure 7.3: Results of a spatial cluster analysis with the program BAPS (Corander et al., 2008) using microsatellite data of 24 populations ($n = 288$) of *Campanula thyrsoides* from the Schynige Platte. Shown is the most likely partition out of 50 independent runs with two clusters. The different clusters 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. 7.1 for locations).

Table 7.2: 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	$F_{ST} = 0.076$***
Among populations within groups	88.4	0.10	5.1	$F_{SC} = 0.052$***
Within populations	913.3	1.78	92.4	$F_{CT} = 0.025$***
Total	1014.0	1.93		

Groups represent the two spatial genetic groups as inferred from Bayesian cluster analyses. Significance was assessed by 5000 permutations. ***P < 0.001.

Table 7.3: Genetic diversity indices, occurrence of private alleles and inbreeding in 24 populations of *Campanula thyrsoides* from the Schynige Platte.

Pop.	H_O[a]	H_E[b]	N_A[c]	N_P[d]	F_{IS}[e]
1	0.839	0.741	5.4	0.0	-0.140
2	0.833	0.757	6.0	0.0	-0.106
3	**0.824**	**0.710**	**4.4**	**0.0**	**-0.161**
4	0.708	0.662	4.8	0.0	-0.071
5	0.700	0.686	5.6	0.0	-0.001
6	0.822	0.724	4.4	0.0	-0.147
7	0.677	0.672	5.0	0.0	-0.010
8	0.608	0.576	4.6	0.0	-0.056
9	0.762	0.689	5.0	0.0	-0.112
10	0.793	0.772	5.6	0.0	-0.029
11	**0.854**	**0.780**	**5.4**	**0.0**	**-0.107**
12	0.777	0.761	5.8	0.0	-0.024
13	0.799	0.778	6.2	0.0	-0.034
14	0.751	0.747	5.6	0.0	-0.006
15	0.743	0.664	4.0	0.0	-0.131
16	0.739	0.712	5.0	0.0	-0.043
17	0.579	0.704	5.0	0.4	0.195*
18	0.677	0.733	5.4	0.0	0.082
19	0.717	0.735	5.2	0.2	0.023
20	0.830	0.678	4.6	0.0	-0.246
21	**0.697**	**0.681**	**3.8**	**0.0**	**-0.037**
22	**0.783**	**0.717**	**4.2**	**0.0**	**-0.096**
23	0.707	0.732	4.4	0.0	0.031
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. Asterisks 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 7.4). [a]Observed heterozygosity (Nei, 1978); [b]Expected heterozygosity (Nei, 1978); [c]Mean numer of alleles per locus; [d]Mean number of private alleles; [e]Inbreeding coefficient (Weir and Cockerham, 1984); $^*P < 0.05$

Genetic diversity and inbreeding

Mean observed and expected heterozygosity across all populations were $H_o = 0.75$ and $H_E = 0.71$, respectively (Table 7.3). 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 7.1). 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).

The mean inbreeding coefficient F_{IS} was 0.049 (Table 7.3). 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 and 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 7.4). 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 colonization (Table 7.3).

Table 7.4: Test for recent genetic bottlenecks in 24 populations of *Campanula thyrsoides* from the Schynige Platte.

Population	SMM[a]	TPM[b]
1	0.31	0.31
2	0.50	0.50
3	0.02*	0.02*
4	0.69	0.69
5	0.98	0.98
6	0.41	0.31
7	0.96	0.92
8	0.89	0.69
9	0.92	0.89
10	0.31	0.31
11	0.03*	0.02*
12	0.59	0.59
13	0.92	0.89
14	0.89	0.59
15	0.11	0.08
16	0.41	0.41
17	0.59	0.31
18	0.41	0.41
19	0.69	0.50
20	0.89	0.89
21	0.02*	0.02*
22	0.02*	0.03*
23	0.08	0.08
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 1000 permutations. [a]Stepwise Mutation Model; [b]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'_{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 7.7). 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 bow (J. Stöcklin, University of Basel, unpublished data; Table 7.5). 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 and 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 7.4), support the hypothesis of a weak gene flow among populations.

Spatial genetic structure

The Bayesian clustering analyses revealed an unexpected splitting of the populations into two spatial genetic groups (Figs. 7.2 and 7.3). 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 7.2) 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.

Table 7.5: 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[a]	F_{ST}[b]	G_{ST}[c]	G'_{ST}[d]
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). [a]Number of populations; [b]Differentiation index (Weir and Cockerham, 1984); [c]Differentiation index (Nei, 1973); [d]Standardized G_{ST} (Hedrick, 2005)

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 (Fig. 7.4). The distant-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 and 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 (Table 7.1; Fig. 7.1).

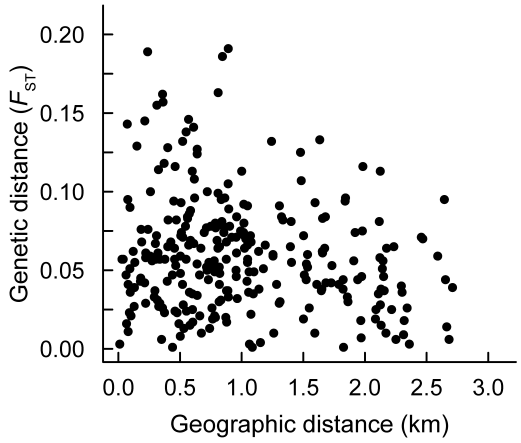

Figure 7.4: Relationship of pairwise genetic differences (F_{ST}) and geographic distances of 24 populations of *Campanula thyrsoides* from the Schynige Platte. The Mantel test for correlation was not significant ($r = 0.22$, $P = 0.99$).

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 colonization, 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 7.4). 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 are more efficient mechanisms for long-distance dispersal of seeds than primary wind dispersal (Morton and 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 et al., 2009). On the Schynige Platte even small populations including 30 or less individuals exhibit high genetic diversity (Tables 7.1 and 7.3). 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 and 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 ($F_{IS} = 0.05$) indicating random mating (Table 7.3). 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 7.4). The presence of bottlenecks is further supported from the absence of private alleles in these populations (Table 7.3). Most likely the detected bottlenecks mirror founder effects during colonization, 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 7.1). Because bottlenecked populations were not inbred (Table 7.3), it suggests that negative effects of small founding populations (Ellstrand and 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 and 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.

Conservation implications

Although we do not consider *C. thyrsoides* endangered on the Schynige Platte, it may be necessary to assist dispersal of this rare bell flower in other regions in the Alps. Such a conservation management strategy for restoration purposes may be needed where its habitats are lost, for example by abandonnement of grasslands previously used as pastures or meadows in the southeastern part of the Swiss Alps (e.g., Rudmann-Maurer et al., 2008).

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 colonization 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

Table 7.6: 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')
Campthy1	$(CA)_{16}$	EF371506	F: CTGCTAGGCTATGCGAGTGTTC
			R: TCTGAATTTGTTGAGAATCTTTTTG
Campthy3	$(CA)_{13}$	EF371506	F: AAAGTTTGATTCCAAGGTGCTC
			R: AAAATAATTCCAGGGACGGAGT
Campthy5	$(CA)_{20}$	EF371506	F: CCAGCGACGCTTTAGTTATTGT
			R: CAAATATAAAGGGGAAGTTACTTATCA
Campthy6	$(CA)_{17}$	EF371506	F: ACAACCTCGAACCAATTTTCAG
			R: CAATTGGGGTCTAACCATTCAC
Campthy9	$(CA)_{24}$	EF371506	F: AATGTCCATGGTGTGGTGAAC
			R: CCATTCAAAGCCGCAGTATTAG

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

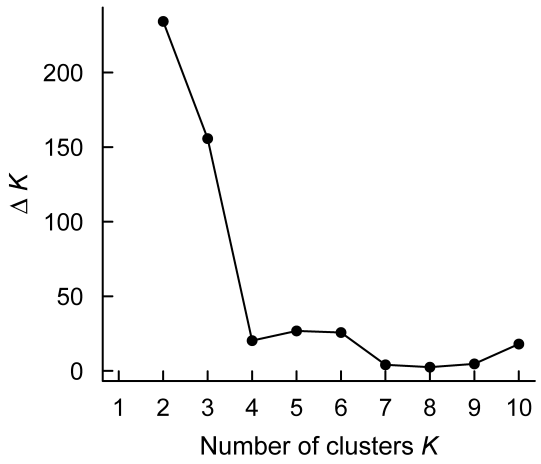

Figure 7.5: The ad hoc statistics (Evanno et al., 2005) of a cluster analysis with the program STRUCTURE (Hubisz et al., 2009) using microsatellite data of 24 populations of *Campanula thyrsoides* from the Schynige Platte. The highest value of ΔK at the true number of clusters ($K = 2$) is shown.

Table 7.7: 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. Pop. = Population

Chapter 8

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

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

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Abstract

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 1000 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.

Keywords: 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 and Roy 2000), the breeding system of the species (Loveless and Hamrick 1984; Hamrick and Godt 1996) and adaptations of seeds or pollen to efficient dispersal (Van der Pijl 1982; Loveless and 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 and 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 and 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 and 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 and 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 and 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?

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 and coworkers (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 10m of 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. 8.1). 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.
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. 8.1) 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 and Hartl 2004). However, Vitalis and co-workers (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.

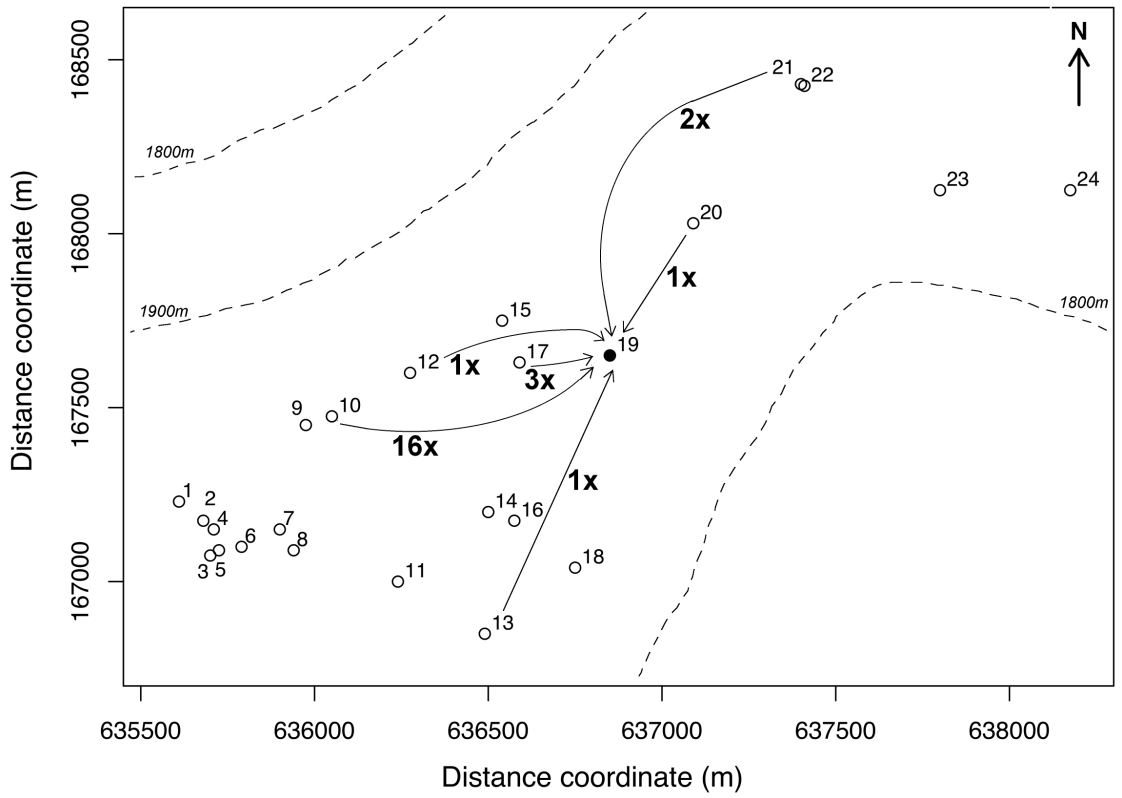

Figure 8.1: Population locations of *Campanula thyrsoides* on the Schynige Platte. Arrows with numbers indicate events of gene flow into the study population (filled circle) based on the rare-allele method using the programs Cervus (Kalinowksi et al. 2007) and GeneClass2 (Piry et al. 2004; see Methods and Table 8.4). Coordinates according to Swiss grid. Isolines are schematic.

Paternity analysis

Sampling design — On August 14th of 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 October 28th of 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$\times$ 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 sec annealing at primer-specific temperatures (Campthy 1, 3, 5, 6, 9: 56 °C; Campthy 15: 60 °C) followed by 30 sec at 70 °C and 30 sec 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 Table 8.5; Bacles and 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 and 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 ($N_{comparison}$) (Table 8.1; Bacles and 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.

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

Table 8.1: 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. $N_{mismatch}$ is the number of mismatching samples and $N_{comparisons}$ is the number of compared samples. Error is the calculated error rate.

	RAW			BIN		
	$N_{mismatch}/N_{comparisons}$	*Error*	*PEP*	$N_{mismatch}/N_{comparisons}$	*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

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 and 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 and 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. 8.1), 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 (Campthy1, 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:

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

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 July 11$^{\text{th}}$ of 2008 and July 13$^{\text{th}}$ of 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 8.1). Paternity exclusion probabilities amounted to 0.990 (Table 8.1). 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 8.2).

Table 8.2: 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). 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.

Assignment class	Definition	N (% of total)
Immigrated	LOD $\leq$ 0	25 (7.6%)
Unassigned, potential gene flow into population	0 < Thr & Δ < 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%)

Within-population pollen movement

The mean pollination distance based on the paternity analysis was 16.2 m (Table 8.3; Fig. 8.2), the average distance of pollinations expected based on random mating was 27.9 m. Therefore, the distribution of realised pollinations showed significantly shorter distances than expected based on random mating (Mann-Whitney *U*-test, Table 8.3; Fig. 8.4). 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. 8.3). These directions showed a distribution being different from random mating (Table 8.3), which predicted pollinations mainly in northeast and southwest directions due to more plants positioned along this axis. Two pollinations were due to self-fertilisation.

Table 8.3: 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*—sample size of detected pollinations; dst—average distance; distr—pollination distribution; dir—pollination direction; $_{pol}$—realised pollination; $_{ran}$—random pollination; Kolmogorov-Smirnov test for differences in shape and location and Mann-Whitney *U*-test for differences in location.

	n	dst_{pol} ± SD (m)	$distr_{pol} \sim distr_{ran}$ Kolmogorov-Smirnov	$distr_{pol} \sim distr_{ran}$ Mann-Whitney *U*	$dir_{pol} \sim dir_{ran}$ 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	$P<0.0001$	$P<0.0001$	$P<0.0001$

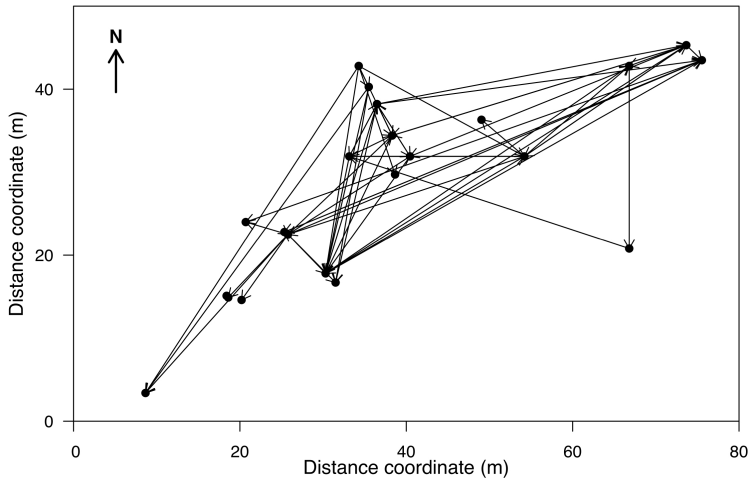

Figure 8.2: Map showing the 22 flowering individuals of *Campanula thyrsoides* in the study population no. 19 on the Schynige Platte in 2007 with arrows indicating pollinations from father to mother as assigned by Cervus (Kalinowksi et al. 2007).

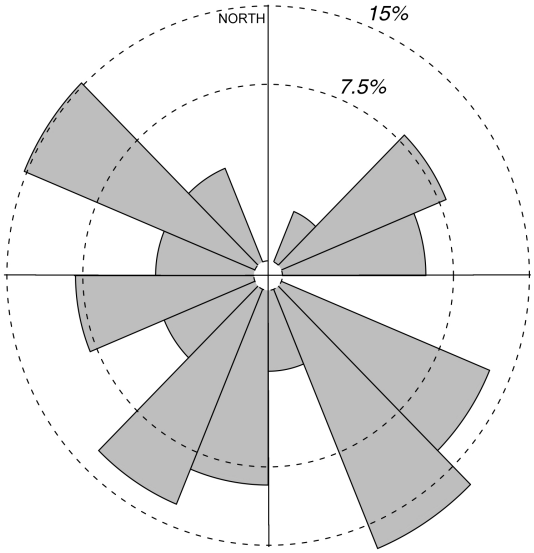

Figure 8.3: Windrose with size of grey areas indicating percentages of pollinations in *Campanula thyrsoides* in specific directions within the study population no. 19 on the Schynige Platte in 2007 as assigned by CERVUS (Kalinowksi et al. 2007).

Reproductive success

For mother plants, the logistic regressions of source diversity index were not significant for distance to the nearest neighbour ($n = 6$; $\text{Chi}^2 = 2.1$; $P = 0.15$) and for the average distance to source individuals ($n = 6$; $\text{Chi}^2 = 3.05$; $P = 0.08$). 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 8.4) and all five first-rank source populations were neighbouring the study population (Fig. 8.1).

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 8.4).

Table 8.4: 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 Methods). 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 method are indicated in bold.

	Rare alleles × relative distance					GeneClass2 × relative distance			
Sample ID	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

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 Fig. 8.5). The realised average distances were 62.1 m for 2008 and 34.0 m for 2009 (Table 8.3). 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 8.3).
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 8.2), 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 and co-workers (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 8.2). 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 and 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. 8.1). 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. 8.1) of which six have been found to contribute pollen in this study (Table 8.4).

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 and co-workers (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 and co-workers (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 exhib-

ited 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 et 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 (Table 8.3; Fig. 8.4). 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 8.3), 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.

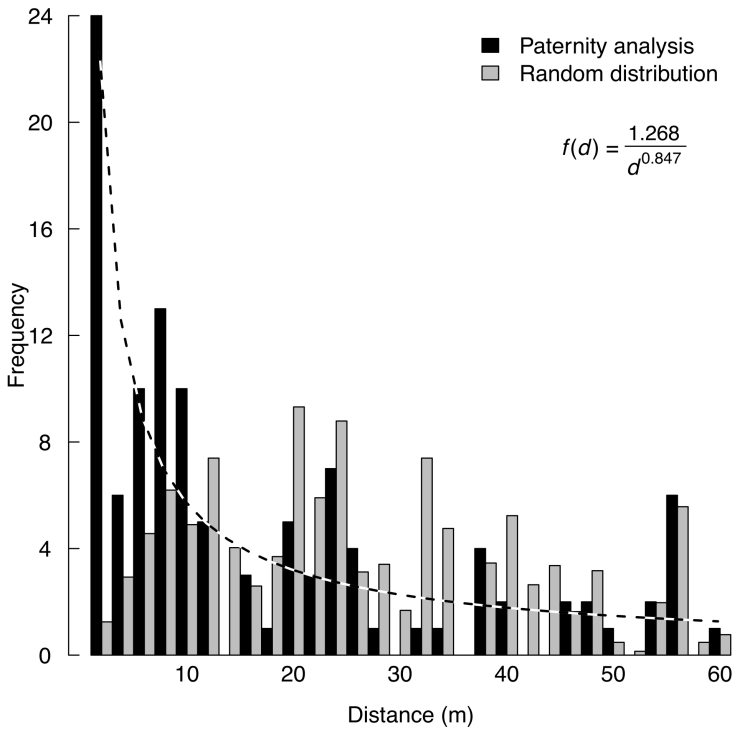

Figure 8.4: Histogram of pollination frequencies in *Campanula thyrsoides* from the paternity analysis (black bars) and from a distribution based on random mating (grey bars) based on distance classes of 2 m. The dotted line indicates the model fit through the paternity analysis data.

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. 8.1) 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 and 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 and 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 8.3). 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 and 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 and 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 and Hamrick 1984; Vitalis 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 and 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 and Hartl 2004). However, the theoretical study by Vitalis and co-workers (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 popula-

tions. 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

Table 8.5: Binning applied to alleles of different *Campanula thyrsoides* loci. Allele codes indicate their lengths in basepairs.

Locus	Alleles binned
Campthy1	160;162
Campthy2	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	1184;186
Campthy15	172;174
Campthy15	176;178

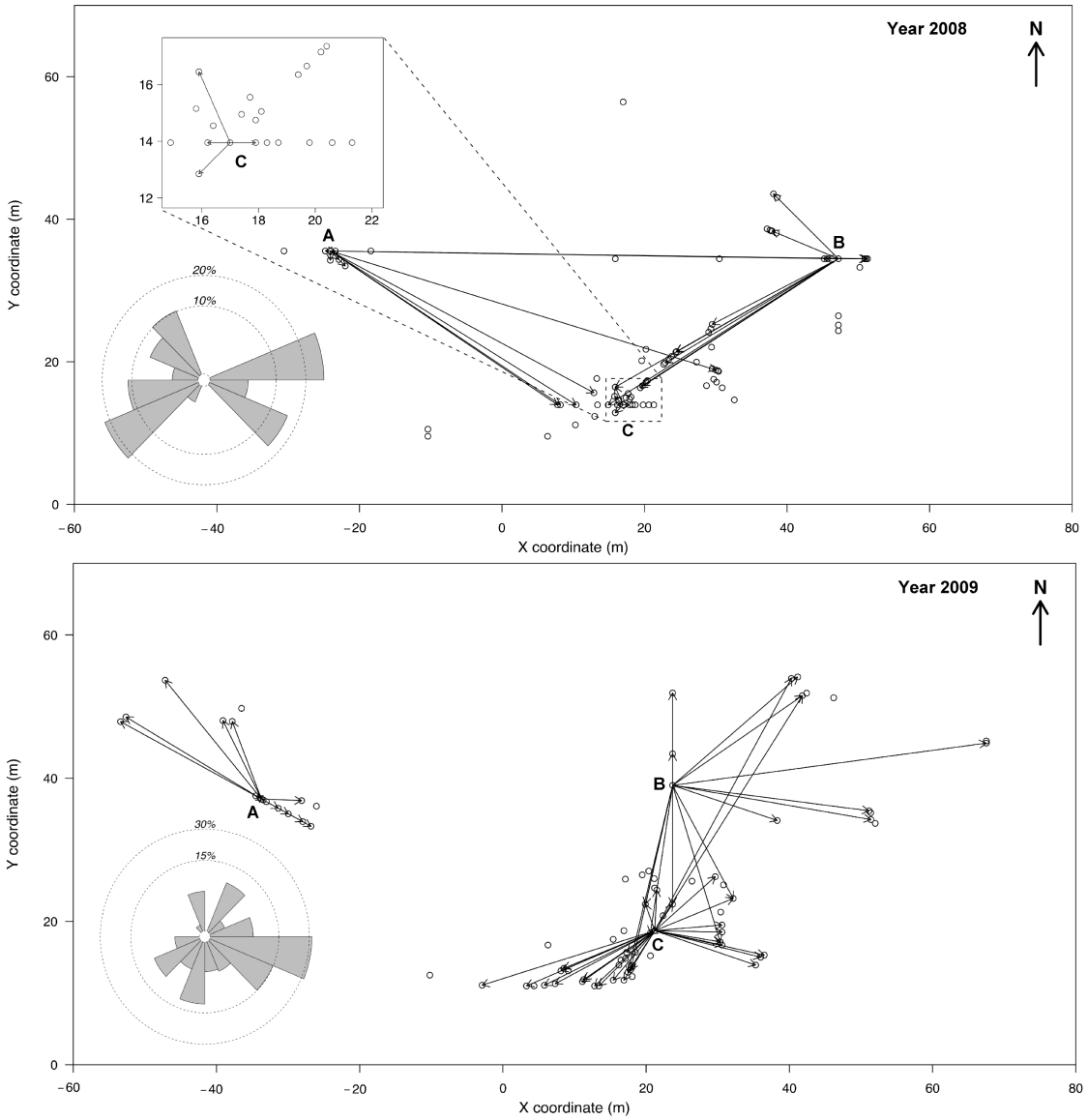

Figure 8.5: Maps showing the pollinations as observed using fluorescent pollen applied to three flowering individuals (A, B, C) each in 2008 (top) and 2009 (bottom). The scale and coordinates of both maps is similar (also to Fig. 8.2). The inset in the map shows a windrose with size of grey areas indicating percentages of pollinations in specific directions.

Chapter 9

Limited colonization potential of a rare Alpine plant as extinction threat

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Limited colonization potential of a rare Alpine plant as extinction threat

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Abstract

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 the colonization of new habitats. We investigated the potential of the rare Alpine plant *Campanula thyrsoides* L. to colonize 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 low to high habitat quality for adult *C. thyrsoides*. In each site we applied a disturbance treatment, added seeds in different densities, and monitored survival of seedlings over two consecutive years. Habitat quality for adult *C. thyrsoides* was not positively related to the number of survived seedlings. 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 assume that *C. thyrsoides* is microsite limited rather than seed limited at the local scale. Microsite limitation is supported by the result that seedling survival of *C. thyrsoides* was enhanced in vegetation gaps created by disturbance. We conclude, that *C. thyrsoides* may get endangered in the future when environmental changes cause local extinctions of populations. Therefore, it could be worthwhile to assist dispersal of this rare Alpine plant to suitable unoccupied habitats and to support seedling recruitment by disturbance.

Keywords: Beals index value, *Campanula thyrsoides*, dispersal limitation, habitat quality, seed sowing experiment, Swiss Alps

Introduction

The occurrence of suitable habitats and the dispersal ability of a plant species are the two main factors that influence a plant's distribution at the regional scale (Münzbergova and 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 successfully recruit seedlings and to establish a fully self-sustainable population in a newly colonized habitat. There is a well known evolutionary trade-off between the number of seeds and seed size: a high seed number increases the chance of dispersal to a distant suitable habitat, while heavier seeds increase the establishment probability of seedlings (see review in Moles and Westoby 2004). At the local scale, distribu-

tion is theoretically limited by the availability of either microsites or seeds (Münzbergova and Herben 2005). However, in nature, local distribution seems to be influenced by a complex interaction of microsite availability, seed limitation and by the interspecific competition among seedlings (Garchìa-Camacho et al. 2010; Egawa and Tsuyuzaki 2011).

In the European Alps, the heterogenous topography of the landscape may limit the dispersal ability of plants (Körner 2003; Bacles et al. 2006). Environmental conditions in alpine habitats are stressful for seedling establishment of plants due to the short time available without a snow-cover. In alpine plant communities, seedling establishment was strongly dependent on the dispersal mode and the successional stage of available sites (Stöcklin and Bäumler 1996; Niederfriniger-Schlag and Erschbamer 2000). In addition, land use changes and global warming has recently led to a loss of suitable habitats for many alpine plant species and to an up-ward shift in the altitudinal range of several species (Walther et al. 2002; Rudmann-Maurer et al. 2008). In a fast-changing environment, species with a weak colonization potential and limited dispersal ability will be the losers of global warming and may need assistence by the dispersal of seeds (Primack and Miao 1992). Therefore, from the perspective of preserving alpine biodiversity, investigating the limiting factors of a species' colonization potential may allow predictions of future range changes in alpine plant species and the formulation of species-specific management strategies for rare plants (e.g. Franz and Eriksson 2003).

In the present study, we investigated the colonization potential and dispersal ability of the rare Alpine plant *Campanula thyrsoides* L. on a mountain plateau (Schynige Platte) in the Swiss Alps. *C. thyrsoides* is best suited to study distribution limitations at the regional and the local scale because for several reasons. Firstly, *C. thyrsoides* has a very narrow ecological niche (Wüest 2008). Therefore, the species has to disperse over long distances to reach new suitable habitats. Secondly, while a single individual plant of this species produces numerous seeds, the dispersal propagules have no morphological structures to support dispersal, and pre-dispersal seed predation can cause complete seed loss (Kuss et al. 2007), suggesting that the distribution of this species is dispersal as well as seed limited. Thirdly, after colonization of a new site, because of the strong self-incompatibility and monocarpic life-cycle of *C. thyrsoides*, at least two individuals must reproduce at the same time in order to successful establish a new population.

We used a relatively new method (Münzbergova and Herben 2004) to identify suitable habitats by using species co-occurrence data from vegetation relevées on the Schynige Platte with and without adult *C. thyrsoides* (Wüest 2008). Based on a classification according to the occupancy and suitability of habitats (Beals index value; Münzbergova and Herben 2004), we selected sites with low to high habitat quality for a seed sowing experiment with *C. thyrsoides*. Seed sowing experiments are a straightforward method for investigating dispersal of plants (Turnbull et al. 2000), and allowed us to test whether germination and survival of seedlings is dependent from the habitat quality 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 efficient management regime for conservation purposes of the rare *C. thyrsoides*. Particularly, we addressed the following hypotheses: (1) The higher the habitat quality (Beals index) for adult *C. thyrsoides* is, the higher is the germination and survival rate of seedlings. (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 regime.

Methods

Study species

Campanula thyrsoides occurs in the European Alps, the Jura Mountains and in the Dinaric Alps on the Balcany (Aeschimann et al. 2004). Subalpine and alpine grasslands to screes on steep slopes and on limestone or carbonate-bearing schists are typical habitats of the species. 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 may 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 on disturbed areas such as road shoulders, where populations may expand locally (Kuss et al. 2008), while it is rare on the regional scale and protected in the majority of the Alpine countries (Moser et al. 2002). *C. thyrsoides* is one of the few Alpine monocarpic perennials. Plants produce a dense spike composed of 50–200 yellow and bell-shaped protandrous flowers (Scheepens et al. 2011). Plants 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 of the mother plant, and there is no evidence for a persistent seed bank in *C. thyrsoides* (Kuss et al. 2007).

Vegetation relevées

As study site, we used the topographically highly structured mountain plateau of the Schynige Platte (1800–2100 m a.s.l.; centered at 46° 39' 12" N; 7° 54' 42" E) in the Swiss Alps. On the Schynige Platte, *C. thyrsoides* occurs in 24 spatially separated populations, mostly in grassland or steep screes. In 2008, 87 vegetation relevées were performed in 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 (Münzbergova and Herben 2004) using vegetation relevées with and without *C. thyrsoides* from Wüest (2008) and 138 relevées from Fischer and Wachter (1991). For each of the 225 relevées, a suitability index (Beals index value; Münzbergova and Herben 2004) was calculated. The value of this index estimates the probability of a species occurrence (*C. thyrosides* in our case) in a distinct habitat. The higher the Beals index is (range 0–1), the more suitable a habitat is for the target species.

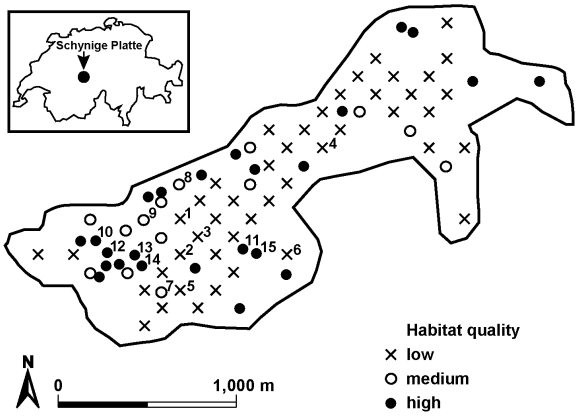

Figure 9.1: Map of the study region on the mountain plateau of the Schynige Platte in the Swiss Alps with habitat quality for *Campanula thyrsoides*. Habitat quality (according to the Beals index, see Table 9.1 and explanation in the text): low = unoccupied and unsuitable sites; medium = unoccupied, but suitable sites; high = occupied and suitable sites for *C. thyrsoides*. The 15 experimental sowing sites used in this study are marked with numbers topleft of their locations. Other sites are from vegetation relevées in 2008

Seed addition and augmentation experiment

In September 2008, mature seeds from 18 different natural populations of *C. thyrsoides* on the Schynige Platte were collected and mixed. On the 20th of September 2008, seeds from this mixture of different populations were sown in 15 experimental sites (Fig. 9.1). Sites were selected in such a way that they covered the entire spectrum of the Beals index as inferred from the vegetation relevées in Wüest (2008) (Table 9.1). Six of the 15 sites were established in habitats where *C. thyrsoides* already natu-

rally occurs and with a high Beals index. In the following text we refer to these sites as habitats of "high quality". Nine sites were established in unoccupied habitats. From the unoccupied habitats, six sites were considered as habitats of "low quality" (i.e. unsuitable based on a threshold value of Beals index), while three sites are referred as habitats of "medium quality" (i.e. suitable).

Within each of the 15 sites, eight experimental plots of 50 cm x 50 cm were established and the following treatments were randomly assigned to them. Three plots were disturbed by clipping the grasses and herbs directly over the ground and by scratching the upper soil level with a three-fingered fork to simulate the activity of small mammals (e.g. Edwards and Crawley 1999). Seeds were added in three different densities (300, 3,000 and 30,000 seeds), each seed density was applied to two plots of which one was disturbed. Two plots were used as control without any seed addition and one of these control plots was disturbed. Before sowing of seeds, we checked for the presence of already established seedlings of *C. thyrsoides* in the plots and removed them when found. Seedlings were counted at three census points during 2009 and 2010: at the beginning of first, at the end of the first and at the end of the second growing season. We also measured the plant diameter of survived seedlings.

Table 9.1: Location and site characteristics of the 15 experimental sowing sites of *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	pH	Ri	Rco (%)	Gco (%)	Fco (%)	Dco (%)
1	0.099	low	167358	636149	1947	E	30	5	48	2	47	5	0
2	0.116	low	167153	636155	1950	NNE	20	5	62	10	42	10	3
3	0.132	low	167254	636254	1941	WNW	30	5	64	10	20	5	5
4	0.169	low	167744	636942	1945	S	35	5	75	1	47	5	7
5	0.171	low	166958	636147	1984	E	25	5	75	10	30	10	5
6	0.186	low	167146	636751	1876	SE	60	5	82	3	35	8	7
7	0.200	medium	166946	636067	2029	ESE	50	5	66	2	45	2	3
8	0.223	medium	167556	636155	1972	SE	30	7	51	15	40	10	2
9	0.251	medium	167322	635953	2040	SE	75	6	66	30	30	5	5
10	0.276	high	167184	635687	1901	SW	65	7	64	20	25	5	1
11	0.293	high	167176	636503	1928	SSE	80	5	64	7	25	8	13
12	0.305	high	167125	635690	1886	WSW	60	8	61	25	20	5	10
13	0.305	high	167143	635881	2005	SW	60	7	44	20	9	3	13
14	0.306	high	167090	635923	1996	WSW	75	7	48	15	28	10	14
15	0.318	high	167125	636547	1908	SE	100	6	70	15	30	5	8

Habitat suitability was determined by means of the Beals index for *C. thyrsoides* from vegetation relevées, each including an area of 49 m^2. Sites were grouped to habitat quality with low = unoccupied and unsuitable sites; medium = unoccupied, but suitable sites and high = occupied and suitable sites for *C. thyrsoides*. Sites are ordered from lowest to highest Beals index. Lat, Latitude (Swiss Grid); Long, Longitude (Swiss Grid); Exp, Exposition (N = North, E = East, 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 (%))

Mixed-effects model analyses

To analyse count and measuring data of seedlings (split-plot design), we used linear-mixed modelling. Linear-mixed modelling allowed us to handle binomial and poisson data in the same framework as normal data, what has the advantage that transformation of the response variables is 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 survived seedlings from the first year (2009) to the number of germinated seedlings as well as the proportion of survived seedlings from the second year (2010) to the number of germinated seedlings was calculated. Since the number of seedlings after

2009 and 2010 showed a poisson error distribution, these variables were also analysed with GLMM models, but with a *log* link function (Crawley 2009). In all GLMM models, 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 (LMM).
The simplest models included the Beals index (i.e. habitat quality), the two treatments *Disturbance* and *Seed density* as well as their interaction as fixed factors, while *Site* was included as a random effect. The sample size was $n = 90$, with 15 sites, 2 $\times$ disturbance and 3 x sowing densities. In the more complex models repeated measure analysis was used, with *Time* (i.e. three census points of measurements) included as an additional fixed factor to account for the time-dependency of the survival of seedlings. In these complex models, the random factors *Site* and *Plot* nested in *Site* were also considered ($n = 270$).
The model parameters were estimated with the *glmmPQL* function in the MASS R-package (Ripley 2005). The significance of the fixed effects was tested with F-tests (Faraway 2005), as recommended for GLMM models with overdispersion in Bolker et al. (2009). *A priori* contrasts were used to test for differences due to different Seed density, assuming a t-distribution. As contrasts, low versus medium seed density and medium versus high seed density were tested. Likelihood ratio tests (Pinheiro and Bates 2000) were performed to test the random effects. For models with only a single random factor, we followed methods used by Crainiceanu and Ruppert (2004). The model assumptions (Pinheiro and Bates 2000) were checked using diagnostic plots constructed with the R-packages *ggplot2* (Wickham 2010) and *lattice* (Sarkar 2009). All models were fitted and tested by means of the statistical package R (R Development Core Team 2009).

Additional analyses

To test for seed limitation, we compared in occupied sites the mean number of seedlings in control plots (without seed addition) with means in experimental plots, by using one-sided t-tests. In order to investigate whether habitat quality is related to environmental variables at the sites, we performed a Pearson's correlation analysis between the Beals index and all site characteristics (Table 9.1) using the statistical package R (R Development Core Team 2009).

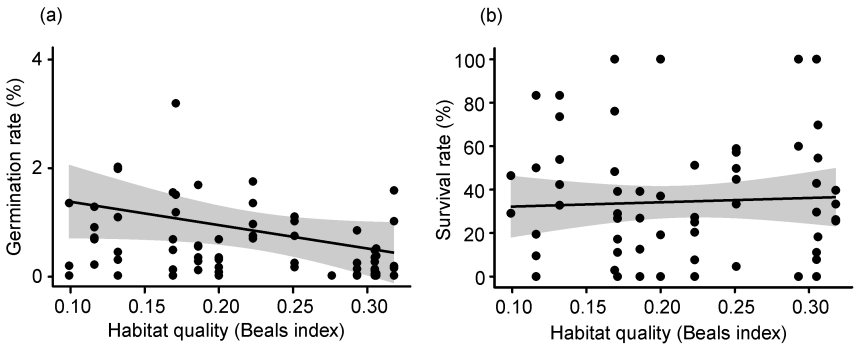

Figure 9.2: Seedling establishment of *Campanula thyrsoides* in a seed sowing experiment on the Schynige Platte with (a) germination rate and (b) survival rate after one year (2009) as a function of habitat quality (Beals index). The lines represent the fit obtained by the linear model analyses and the grey shaded areas are the 95% confidence intervals

Table 9.2: Summary of Generalized Linear Mixed Models (GLMM) testing the effect of habitat quality, disturbance treatment and seed addition density on germination, survival and plant diameter 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			Survival by 2010		
Beals index	1	10.83**	Beals index	1	0.47
Disturbance	1	12.53***	Disturbance	1	6.49*
Seed density	2	2.99(*)	Seed density	2	0.34
Low versus medium		-0.33	Low versus medium		0.57
Medium versus high		-2.43*	Medium versus high		1.09
Disturbance:Seed density	2	1.83	Disturbance:Seed density	2	0.87
Site	1	17.35****	Site	1	3.97*
Survival by 2009			Plant diameter		
Beals index	1	0.13	Beals index	1	0.90
Disturbance	1	4.23*	Disturbance	1	13.0**
Seed density	2	1.1	Seed density	2	4.19*
Low versus medium		1.07	Low versus medium		0.62
Medium versus high		1.57	Medium versus high		2.75**
Disturbance:Seed density	2	1.87	Disturbance:Seed density	2	0.46
Site	1	0.82	Site	1	6.35**

Test statistics are F-values for the fixed effects (*Beals index*, *Disturbance*, *Seed density*, *Disturbance:Seed density*), t-values for *a priori* contrasts, and ChiSquare values for random effects (*Site*). The Beals index is a measure for habitat quality (for details see Materials and methods). $n = 90$. Asterisks represent significance levels: (*)$P < 0.08$;*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

Results

Effects of habitat quality and time

The habitat quality measured by the Beals index had a significant negative effect on germination rate, the number of survived seedlings, but not on survival rates after one and two years (Tables 9.2 and 9.3). Germination rate was lowest (mean 0.18%; Table 9.4) in sites of high habitat quality and increased with increasing habitat quality (Fig. 9.2a). In contrast, the survival rate after one year (2009: 39.92%) and after two years (2010: 17.40%) was higher in sites of high habitat quality (Table 9.4), but this difference was not significant (Fig. 9.2b, Table 9.2). Time had a much stronger negative effect on the number of seedlings than habitat quality (Table 9.3). Plant diameter of seedlings was not different among sites of different habitat quality (Tables 9.2 and 9.4). Finally, the Beals index correlated positively with slope, pH, rock cover and cover of dwarf shrubs, and negatively with grass cover (Table 9.5).

Table 9.3: Summary of Generalized Linear Mixed Models (GLMM) testing the effects of Beals index, time, disturbance treatment and seed addition density on number of seedlings of *Campanula thyrsoides* in a seed sowing experiment

Source of variation	Model effects	
	df	Test statistics
No. of seedlings		
Time	2	580.03****
Beals index	1	8.20*
Disturbance	1	3.31(*)
Seed density	2	46.09****
Low versus medium		-4.87****
Medium versus high		11.42****
Disturbance:Seed density	2	0.71
Site	1	27.52****

Test statistics are F-values for the fixed effects (*Beals index*, *Disturbance*, *Seed density*, *Disturbance:Seed density*), t-values for *a priori* contrasts, and ChiSquare values for random effects (*Site*). df, degrees of freedom. Time represents a factor of three different census points of measurements. The Beals index is a measure for habitat quality (for details see Materials and methods). $n = 270$, with 90 plots nested in 15 sites. Asterisks represent significance levels: (*)$P < 0.08$; *$P < 0.05$; ****$P < 0.0001$

Effects of disturbance

Disturbance had the strongest positive effect on all measured variables with the exception of the number of seedlings (Tables 9.2 and 9.3). In disturbed plots, germination rate, survival rates, plant diameter of seedlings and the number of seedlings were always higher compared to undisturbed plots (Figs 9.3 and 9.4, Table 9.4). 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.

Table 9.4: Means (SE) for germination rate, survival rates and seedling diameter from a seed sowing experiment with *Campanula thyrosides* on the Schynige Platte. Means were calculated for the habitat quality, disturbance treatment and seed addition density separately

	Habitat quality (Beals index)		
	low	medium	high
Germination rate (%)	1.22 (0.32)	1.36 (0.52)	0.18 (0.06)
Survival rate by 2009 (%)	32.85 (5.41)	31.53 (6.54)	39.92 (7.48)
Survival rate by 2010 (%)	12.94 (3.00)	13.21 (4.45)	17.40 (6.03)
Plant diameter (mm)	5.9 (0.4)	4.0 (0.3)	5.4 (0.4)
	Disturbance		
	undisturbed	disturbed	
Germination rate (%)	0.73 (0.24)	0.93 (0.25)	
Survival rate by 2009 (%)	26.59 (4.60)	41.07 (5.25)	
Survival rate by 2010 (%)	8.00 (2.44)	19.52 (3.77)	
Plant diameter (mm)	4.5 (0.3)	5.8 (0.3)	
	Seed density		
	300	3,000	30,000
Germination rate (%)	1.18 (0.39)	0.77 (0.33)	0.54 (0.12)
Survival rate by 2009 (%)	27.81 (9.36)	31.76 (6.12)	39.96 (4.58)
Survival rate by 2010 (%)	10.42 (5.67)	13.54 (4.12)	16.84 (3.43)
Plant diameter (mm)	5.1 (0.6)	4.6 (0.3)	5.6 (0.3)

Habitat quality (according to the Beals index, see Table 9.1): low = unoccupied and unsuitable sites; medium = unoccupied, but suitable sites; high = occupied and suitable sites for *C. thyrsoides*. Seed density; number of added seeds. $n = 90$

Table 9.5: Correlation analysis of the Beals index (habitat quality) with site characteristics of 15 experimental sites used in a seed sowing experiment with *Campanula thyrsoides* on the Schynige Platte

	r	df	t
Slope (%)	0.84	13	5.58****
pH	0.70	13	3.54**
Species richness	-0.20	13	-0.72
Rock cover (%)	0.61	13	2.80*
Grass cover (%)	-0.62	13	-2.81*
Cover of Fabaceae (%)	-0.13	13	-0.49
Cover of dwarf shrubs (%)	0.66	13	3.18**

r, Pearson's correlation coefficient; df, degrees of freedom; Species richness, number of species assessed in vegetation relevées. $n = 15$. Asterisks represent significance levels: $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$

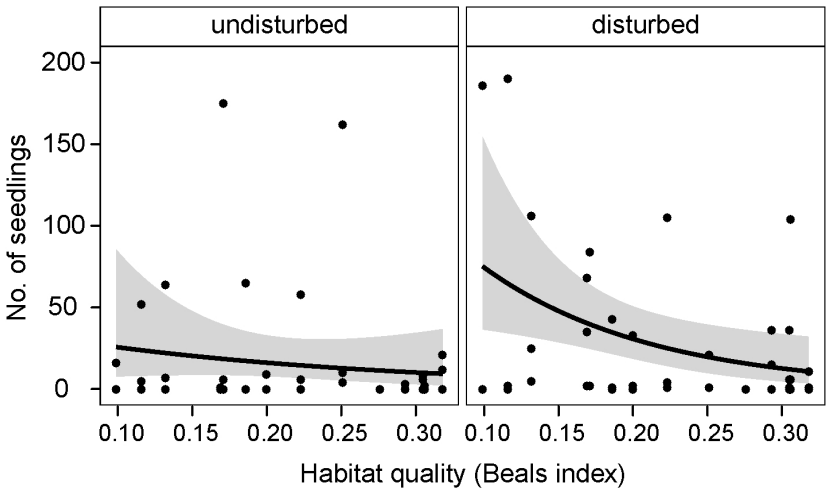

Figure 9.3: Number of seedlings after one year (2009) in a seed sowing experiment with *Campanula thyrsoides* on the Schynige Platte as a function of disturbance treatment and habitat quality (Beals index). The lines represent the fit obtained by the generalized linear model analyses and the grey shaded areas are the 95% confidence intervals

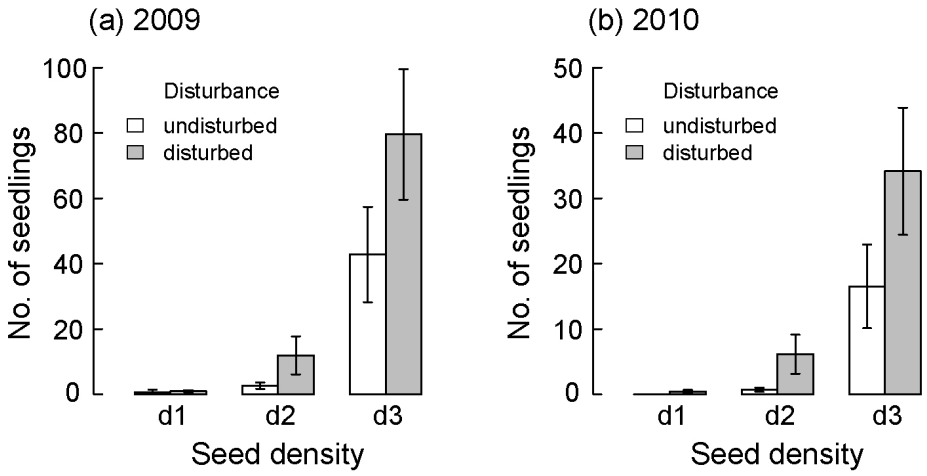

Figure 9.4: The effect of seed addition density and disturbance treatment on the number of seedlings after (a) one year (2009) and after (b) two years (2010) in a seed sowing experiment with *Campanula thyrsoides* on the Schynige Platte. Seed density: d1; 300 seeds, d2; 3,000 seeds, d3; 30,000 seeds

Effects of seed density

The number of added seeds significantly affected germination rate, plant diameter and the number of seedlings, but not survival rates (Tables 2 and 3). The number of seedlings increased with increasing seed density (Fig. 9.4). Contrasts tests indicated that the number of seedlings was different ($P < 0.0001$) between plots of low and medium, and of medium and high seed density (Table 9.3).

The number of seedlings in control plots (mean = 5.1) was neither significantly different from that observed in plots in which seeds were added in high density (mean = 9.0; t = -1.14, df = 11, $P = 0.28$), nor in plots of medium seed density (mean = 1.2; $t = 1.13$, df = 11, $P = 0.28$) or plots of low seed density (mean = 0.1; $t = 1.45$, df = 11, $P = 0.18$).

Discussion

Habitat quality and seedling establishment

Germination rate and the number of seedlings in *C. thyrsoides* were significantly lower in experimental sites of a high habitat quality measured by the Beals index. This negative relationship contradicts our hypothesis and results from another study with *Succisa pratensis*, where habitat quality correlated positively with the number of seedlings (Milden et al. 2006). In a long-term study that investigated the effects of habitat quality 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). Therefore, 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 sites of only low or medium suitability (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 were not different between sites of different habitat quality (Table 9.4).
Our results therefore suggest, that the Beals index is only valid for habitat requirements of adult *C. thyrsoides*, based on which habitat quality was assessed (Wüest 2008). We know from integral projection models, that populations of *C. thyrsoides* can moderately increase and persist even at an extremely low establishment rate of seedlings (Schynige Platte: seedling establishment rate $< 0.1\%$; Kuss et al. 2008). Therefore, it is likely that habitat conditions for successful seedling recruitment in natural populations have changed since colonization due to the ongoing vegetation succession. We assume that *C. thyrsoides* needs open and newly created habitats to establish new populations and that after a population has been established, it can persist for a long time, even when the habitat is changing. The habitat quality measured by the Beals index may not represent the suitability of a habitat for colonization by seedlings, but rather the probability of a habitat to be occupied by an already established population.
The correlation analysis further supports that seedling recruitment of natural populations may suffer from vegetation succession. Occupied sites of high habitat quality had an increased cover of dwarf shrubs, while in unoccupied sites with a lower Beals index, the vegetation was dominated by grasses (Tables 1 and 5).

Limitation at the regional scale

From vegetation relevées on the Schynige Platte we know that habitat limitation is not an important factor for the actual distribution of *C. thyrsoides*, since only a small fraction (26%) of all suitable habitats for *C. thyrsoides* on the Schynige Platte are occupied by the species (Wüest 2008). Therefore and because seeds lack morphological structures for wind or animal dispersal (Kuss et al. 2007), we hypothesized 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 even might be a common feature for alpine plants (Erschbamer et al. 2008). However, extending the recording of seedling survival in our experiment over more than two years would provide better estimates of dispersal limitation, since only long-term data could show whether seedlings in unoccupied sites would lead to a sustainable population (Gustafsson et al. 2002).

Limitation at the local scale

Our hypothesis that the distribution of *C. thyrsoides* at the local scale is fully seed limited must be rejected, since augmentation of seeds in already occupied sites resulted not in a higher number of seedlings compared to control plots. Therefore, the already established population of *C. thyrsoide*s produces enough seeds to ensure the local spread of *C. thyrsoides*. Consequently, we must accept the alternative hypothesis that

C. thyrsoides is at least partially microsite limited. Microsite limitation is supported by the result of the disturbance treatment that was applied (Figs 9.3 and 9.4). Disturbance increased the number of seedlings considerably, probably as a consequence of created vegetation gaps, increased light availability and lower competition. Furthermore, in a demographic study (Kuss et al. 2008), the average germination rate in a natural population on the Schynige Platte was estimated in permanent plots to be 0.078%, while in a greenhouse study (Ægisdóttir et al. 2007) germination of *C. thyrsoides* on wet filter paper was much higher with 75%. Therefore, 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* (Kuss et al. 2008), increasing the seedling establishment rates resulted in a dramatically increase of population size. But seed predation could still play a significant role for the local distribution of *C. thyrsoides* (Kuss et al. 2007), since pre-dispersal seed predation is a crucial factor affecting seed availability (Juenger and Bergelson 2000; Szentesi and Jermy 2003; Orrock et al. 2006).

Conservation implications

Summarizing our results, the species *C. thyrsoides* may get endangered on the Schynige Platte in the future because of its weak colonization potential in combination with its narrow ecological niche, monocarpic life-cycle and strong self-incompatible breeding system. Genetic diversity, however, was generally high in all populations in this region (Frei et al. submitted) and inbreeding depression may not be a problem. Nevertheless, we consider the currently established natural populations of *C. thyrsoides* as threatened, when the present availability of suitable habitats will continue shrinking because of global warming. Shifts in the distribution of alpine plants in response to increased summer temperatures have been reported repeatedly (Grabherr et al. 1994; Walther et al. 2002; Parmesan and Yohe 2003). The expected migration of plants because of global warming is likely to be related to their different dispersal abilities (le Roux and McGeoch 2008). Therefore, we suggest that particularly dispersal limited species like *C. thyrsoides*, will be the losers of climate change.

A long, traditional management history is obviously important for the persistence of many species (e.g. Eriksson 1998) and for *C. thyrsoides* in particular, since suitable habitats are abundantly available on the Schynige Platte (Wüest 2008). Therefore, another putative risk for the population persistence of *C. thyrsoides* would be changes in the current land use practises (Körner 2003). For several centuries and to this day, the area of the Schynige Platte is used as summer pasture for cattle (Lüdi 1948). Pastures, which are more heterogeneous and characterized by light gaps from large-scale disturbances by grazing animals, favours the establishment of seedlings much more than homogeneous mown grassland does (Bullock et al. 1995; Coulson et al. 2001). But it seems, that such disturbances by cattle are not enough for the establishment of new populations at suitable unoccupied sites, especially since *C. thyrsoides* often grows at steep slopes where cattle is absent, what could explain the dominance of dwarf shrubs at these slopes. An efficient management strategy for conservation of the rare and strongly dispersal limited *C. thyrsoides* at the regional scale might therefore only be to assist dispersal of this species to suitable habitats (Primack and Miao 1992) and to prevent succession by a sustainable land use management.

At the local scale, a small-scale disturbance regime would be worthwhile during the first years after sowing, which has been shown to increase seedling establishment in *C. thyrsoides* (Table 9.2), as well as in other grassland species (Klinkhamer and De Jong 1988). Such artificially disturbed sites are best suited for germination and early survival in *C. thyrsoides*, since more than 180 seedlings per m^2 were naturally growing in such a site (road shoulder to Furka Pass, personal observation).

Conclusions

Our results suggest that the habitat quality 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 Alpine regions. Therefore, rare species like *C. thyrsoides* with a weak colonization potential will be disadvantaged when habitats will continue shrinking and no species-specific management strategies are planned, as we proposed in the present study.

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Chapter 10

Glacial history and local adaptation explain differentiation in phenotypic traits in the Alpine grassland herb *Campanula barbata*

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Glacial history and local adaptation explain differentiation in phenotypic traits in the Alpine grassland herb *Campanula barbata*

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Abstract

The glaciations during the Quarternary affected the spatial distribution of neutral genetic variation in many widespread Alpine plants. Long-term survival of plants in isolated refugia on the fringes of the European Alps is proposed as the main driver for molecular differentiation across the Alps. We hypothesize that glacial history may well have had effects on phenotypic differentiation in accordance with molecular patterns. Using a common garden experiment with 15 populations from across the Alps, we found that two phylogeographic lineages of the subalpine grassland plant *Campanula barbata* are differentiated in vegetative and reproductive traits. This indicates that glacial history affected phenotypic differentiation, whether through neutral processes or adaptation. Furthermore, correlations of number of leaves, plant height and aboveground biomass with environmental conditions at population origins indicated that part of the observed differentiation among populations is due to local adaptation to current conditions. These traits decreased with increasing elevation, likely as a result of adaptation to colder conditions. Our results indicate that phenotypic differentiation across the investigated regions is due to glacial history-related processes as well as due to more recent processes of adaptation.

Keywords: common garden, elevation, European Alps, genetic drift, phylogeography

Introduction

Glacial history affected the phylogeographic structure of many widespread Alpine plant species (Schönswetter et al. 2005; Parisod 2008; Paun et al. 2008; Alvarez et al. 2009; Thiel-Egenter et al. 2009). It is generally assumed that specific genetic signatures evolved due to long-term survival in isolated refugia outside the Alps (Schönswetter et al. 2005) or on so-called nunataks, i.e. ice-free habitat on mountain peaks (Stehlik 2000). Putative refugia for Alpine species have been located around the Alps (Stehlik 2000; Tribsch and Schönswetter 2003; Schönswetter et al. 2005), but each species has its own particular glacial history (Hewitt 1996; Stewart et al. 2010, Kuss et al. 2011).
Phylogeographic patterns are commonly detected with the use of putatively neutral molecular markers. Such markers can reveal past processes of mutation, gene flow and neutral genetic drift, the latter as a result of finite population size, bottlenecks and founder effects (Nei et al. 1975). At the time-scale of glaciations (c.

100,000 yrs), mutations and neutral genetic drift can play a considerable role in differentiation (Hartl and Clark 1995; Klekowski 1997; Schönswetter et al. 2005), whereas founder effects are likely during remigration into deglaciated areas of the Alps as Ice Ages are known to cease abruptly (Gugerli et al. 2001; Hewitt 2004).

At the level of the phenotype, trait differentiation among populations can either be caused by stochastic processes or by selection (Lande 1976). Though any phenotypic differentiation could be solely due to stochastic processes, it is likely that populations adapted to the environmental conditions prevailing in the refugia or during remigration (Hewitt 1996). For instance, survival of the alpine plant *Campanula thyrsoides* in refugia in the temperate north versus the submediterranean southeast of the Alps is presumed to have led to allopatric subspeciation (Kuss et al. 2011; Scheepens et al. 2011). Thus, glacial history could have led to phenotypic differentiation, and this could be a result of neutral processes as well as differential adaptation.

Few studies are available that investigate effects of glacial history on phenotypic differentiation in Alpine plants. At the beginning of the previous century, Brockmann-Jerosch (1908) drew the first conclusions on the effects of glacial history on the origin of related species, subspecies and varieties in the European Alps based on observations of their current distributions (Holderegger et al. 2011). More recently, Ozenda (1988) reviewed and summarised important generalisations on plant distributions and plant origin in relation to glacial history. More recently, studies on *Campanula thyrsoides* (Scheepens et al. 2010; Kuss et al. 2011; Scheepens et al. 2011) and *Geum reptans* (Frei et al., unpublished) showed considerable phenotypic differentiation in vegetative, reproductive and phenological traits among phylogeographic groups defined from molecular marker analysis. Part of the observed variability could be explained by adaptation to current climatic conditions, as can be expected since the Alpine landscape is spatially and temporally very heterogeneous (Körner 2003).

In this study we investigate phenotypic differentiation in *Campanula barbata*, a subalpine-alpine grassland herb that is widespread in the European Alps. Thiel-Egenter and co-workers (2011) reported a strong phylogeographic break line for this species in the Austrian Alps (robustness 61-100%), running north-south through Tyrol (c. Munich–Innsbruck–Bolzano–Venice). In Switzerland, a second break line was detected running from Lucerne to Lugano (robustness 21-80%). West of this second break line, a couple of weaker break lines were found (21-60%; Thiel-Egenter et al. 2011), suggesting that multiple refugia have formed these smaller groups which subsequently may have formed suture zones with increased levels of genetic diversity (Petit et al. 2003; Gugerli et al. 2008). Indeed, floristic and geological biogeographic data indicates that this area in the Western Alps shows a high number of putative refugia (Stehlik 2000; Parisod and Besnard 2007). The two strong break lines in Tyrol and Switzerland mentioned above are congruent with large-scale biogeographic distribution patterns based on floristic data (Merxmüller 1952, 1953, 1954; Ozenda 1988), although the most notable biogeographic break line based on floristic data, dividing the Swiss Alps from Bodensee to Lake Como, appears absent in *C. barbata* and other species such as the Campanulaceae *Phyteuma betonicifolium* (Alvarez et al. 2009), the sedge *Luzula alpinopilosa*, the Asteraceae *Hypochaeris uniflora* (Thiel-Egenter et al. 2011), and the two Rosaceae species *Geum reptans* (Frei et al., unpublished) and *G. montanum* (Thiel-Egenter et al. 2009).

By means of a common garden experiment (Clausen et al. 1948), we compare vegetative and reproductive traits of *C. barbata* plants from 15 populations from the west and the east of this second break line in Switzerland (Lucerne–Lugano). We hypothesise that phenotypic differentiation is present between the two groups of populations from the western and central Alps, and that the phenotypic differentiation is retained when adjusted for distance effects between the sampled populations. We also investigated the response of the study plants to clipping, as *C. barbata* occurs in natural and managed grasslands (Aeschimann et al. 2005). We further hypothesise that part of the observed phenotypic differentiation is due to adaptation to current environmental conditions, as the Alpine landscape exhibits strong spatial and temporal heterogeneity in abiotic conditions

(Körner 2003). We test this hypothesis by correlating trait values measured in the common garden with climatic conditions at the sites of origin.

Methods

Study species

Campanula barbata L. (Campanulaceae) is a blue, hairy bell flower occurring on acidic soils in relatively nutrient-poor subalpine and alpine grasslands (typical syntaxon: Nardion strictae) and open forests (Lauber and Wagner 2001; Aeschimann et al. 2005). It is widely distributed in the European Alps with additional scattered occurrences 100 km north of Oslo, Norway, and northwest of Ostrava, Czech Republic (Meusel and Jäger 1992). Plants grow as rosettes and, from June till August, produce zero to several inflorescences of 10-40 cm height each bearing two to twelve flowers (Aeschimann et al. 2005).

Common garden experiment

Seeds from three to eight individuals (seed families) were sampled in each of 15 populations, four in the Western Alps (WA) and eleven in the Central Alps (CA; see Introduction, Table 10.1 and Fig. 10.1). The particular division in these two regions was chosen based on the genetic structure revealed by Bayesian clustering analysis of AFLP data (Thiel-Egenter et al. 2011).

From September 2007 on, we germinated seeds on moist filter paper in Petri dishes in a greenhouse located in Basel, Switzerland (276 m a.s.l.). We planted eight seedlings per seed family into pots of 4 cm diameter filled with low-nutrient soil (Anzuchterde, Ökohum, Herrenhof, Switzerland). After 10-18 weeks, plants were repotted into bigger pots (10 × 10 × 10 cm) with potting soil (Topferde, Ökohum, Herrenhof, Switzerland). When necessary, we sprayed insecticide (Traunem®, BioControl, Andermatt; Basudin® Extra, Novartis Agro, Dielsdorf, Switzerland) to control Aphidoidea and Sciaridae outbreaks. Fertiliser (Wuxal®, Maag, Düsseldorf) was added once when growth decelerated. Two weeks before final transplantation to the field site, plants were transferred outside the greenhouse to acclimatise. During this period, anti-snail grains (Ferramol®, BioControl, Andermatt) were applied to limit snail grazing.

On 8 June 2008, plants were transplanted to a common garden located in Davos, Graubünden, Switzerland (N 46°47'06.97", E 9°48'57.02") at 1530m a.s.l. A total of 622 plants were transplanted into freshly ploughed soil, formerly used as an organically fertilised subalpine meadow-pasture. Not all populations had the same number of seed families and replicates within seed families (Table 10.1) due to missing samples and mortality in the greenhouse. Annual precipitation at the common garden location amounts to 1026 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). Regular weeding was necessary to limit interspecific competition. The experimental site was fenced with electric wire with mesh size 15 × 15 cm.

Seven weeks after transplantation, on 29 July 2008, we clipped half of the plants of each seed family to simulate herbivory. All leaves were cut away with scissors as close as possible to the rosette center without injuring the apical meristem. Next summer, on 2 June 2009, the rosette diameter and the number of leaves were determined for each plant. On 19 August 2009, the plant height (i.e. approximate length of inflorescences) was measured and the above-ground biomass was harvested and weighed after drying for 72 hours at 60 °C in a drying oven, after which the number of inflorescences and flowers were counted.

Table 10.1: Location, geographic coordinates (WGS 84) and altitude (m a.s.l.) of 15 sampled *Campanula barbata* populations across the European Alps. Pop—Population abbreviation (see Fig. 10.1), Region—Phylogeographic region: *WA* Western Alps, *CA* Central Alps, *n*—sample size of individuals used in the common garden, *sf*—number of seed families used in the common garden.

Location	Pop	Region	Northing	Easting	Altitude	*n*	*sf*
Col du Lauteret	LAU	WA	45°02'03"	6°23'60"	1900	46	6
Col du Galibier	GAL	WA	45°03'13"	6°24'18"	2100	40	5
Petit St. Bernard	PSB	WA	45°40'17"	6°52'28"	2149	28	4
Grand St. Bernard	GSB	WA	45°51'52"	7°09'32"	2246	36	5
Fiescheralp	FIE	CA	46°24'48"	8°06'15"	2183	24	3
Furkapass	FUR	CA	46°34'39"	8°25'17"	2440	32	4
Tiefenbach	TIE	CA	46°35'41"	8°27'26"	2124	48	6
Steinlimigletscher	STE	CA	46°44'50"	8°27'54"	1908	48	6
Val Fex	FEX	CA	46°23'12"	9°46'53"	2032	32	4
Scalettagletscher	SCA	CA	46°43'01"	9°55'29"	2047	64	8
Vadret da Cambrena	CAM	CA	46°24'17"	10°00'06"	2338	25	4
Val Tuoi	TUO	CA	46°47'48"	10°08'28"	1977	47	6
Nauders	NAU	CA	46°52'54"	10°29'21"	1797	56	7
Obergurgl	OBE	CA	46°50'52"	11°01'08"	2260	48	6
Tuxertal	TUX	CA	47°05'46"	11°39'46"	2036	48	6

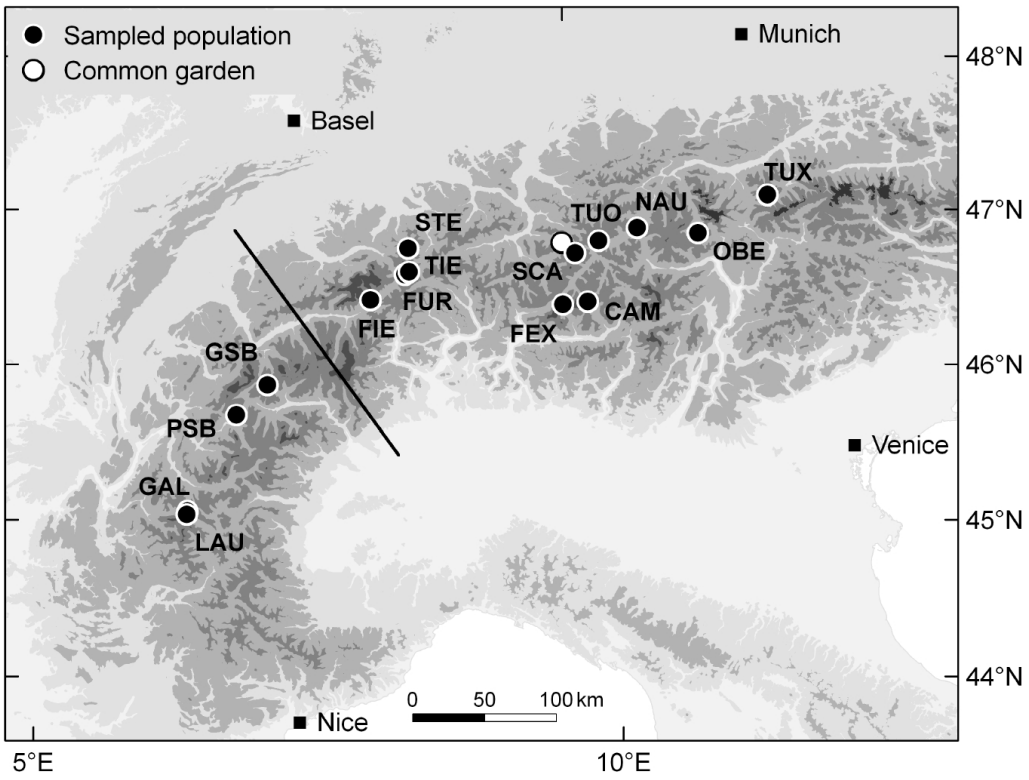

Figure 10.1: Map showing the locations of the sampled populations and of the common garden in the European Alps. See Table 10.1 for abbreviations and coordinates of populations. The line demarcates the Western Alpine populations from the Central Alpine populations. Map projection: Mollweide, Geographic coordinates: WGS 84.

Linear mixed models

All measured traits were analysed using linear mixed-effects models (Crawley 2007) with Type I sums of squares. The clipping treatment was included as a fixed effect in all models, followed by region (fixed), population (random) and seed family (random) which were nested in each other. Interactions between the clipping treatment and region (fixed), population (random) and seed family (random) followed the full factors and were also nested.
To test the significance of model factors, Chi^2-values and P-values were derived from likelihood ratio tests of model comparisons using maximum likelihood. We started with deletion of the interactions and climbed up until all factors had been tested. To calculate variance components, we performed models with all factors treated as random (Bates 2005; Crawley 2007).
Since geographic distances between populations within and among the two phylogeographic regions are variable, a significant region effect must not necessarily be due to phylogeographic region but can be caused by distance-related differentiation. Such distance-related differentiation could be caused by neutral genetic drift due to isolation by distance or by local adaptation to distance-related gradual change in environmental conditions. Therefore, we performed a second set of models with a covariate of euclidean distance of populations to the common garden in order to adjust for such potential effects of distance-related differentiation. A significant effect of phylogeographic region in these models is stronger evidence for an effect on phenotypic differentiation due to glacial history.
Homogeneity of variances and normality of model residuals were checked visually by constructing diagnostic plots. In order to improve normality of model residuals, data of aboveground biomass and number of flowers were logarithmised (Crawley 2007). Errors of all count data, including number of inflorescences, fitted a normal distribution better than a Poisson distribution. The sample size for number of flowers was low (275), and only 28 out of 80 seed families had 2 or more samples in both control and clipped treatments, which could preclude any rigorous testing of the interaction between treatment and seed family.

Adaptation

We performed Pearson's correlations between population mean trait values of unclipped plants, calculated from seed family means, and five environmental factors at the location of origin: (i) altitude of population origin (Table 10.1), (ii) precipitation, and (iii) minimum, (iv) mean and (v) maximum temperature, based on monthly averages over 50 years (WorldClim data; Hijmans et al. 2005; www.worldclim.org). We additionally tested whether any correlations with altitude remained when the region effect was statistically removed. Simple ANOVAs of population mean trait values with region as explanatory variable were performed and the residuals were then used to test for correlations with altitude of origin.
We did not correct for multiple testing of correlations (e.g. Holm-Bonferroni correction; Holm 1979), since this would strongly affect modestly statistically significant results (Moran 2003). All analyses were performed using R statistical package (R Development Core Team 2009; version 2.10.1) with package „lme4“ for analysing linear mixed models (Bates and Maechler 2009).

Results

Phenotypic differentiation

Significant differentiation between the two regions was present in plant height and number of inflorescences (Table 10.2), which explained 15.6% and 3.3% of total variation, respectively. Plants from the Western Alps were taller but had fewer inflorescences than plants from the Central Alps (Fig. 10.2c–d). The remaining traits were not significant and did not explain any variation for region.
Clipping significantly reduced all trait values except for plant height (Table 10.2, Fig. 10.2). This effect of clipping was strongest for number of flowers and biomass (Fig. 10.2e–f), which were reduced with 47% for WA and 21% for CA in the number of flowers, and with 35% for WA and 17% for CA in biomass.
Populations within regions were significantly different for all traits and explained more of the variation in plant height than region did (Table 10.2). Seed families were significantly different for rosette diameter, number of leaves, plant

height and number of inflorescences, but not in number of flowers and biomass; the explained variation was highly variable from trait to trait. Interactions with the clipping treatment were never significant, indicating that regions, populations and seed families responded equally to clipping.

The models which included distance to the common garden as initial covariate in order to remove any distance-related pattern of differentiation yielded significant region effects ($P < 0.05$) for all investigated traits except for number of inflorescences (Table 10.3). This suggests that most traits comprise sharp regional boundaries concurrent with the phylogeographic lineages. The distance to the common garden itself significantly explained variation in rosette diameter, number of leaves, plant height and number of inflorescences.

Mortality was low throughout the experiment. Only eleven individuals died between transplantation and the clipping treatment, and no additional plants died during that growing season. Over winter, until the measurement of 2 June 2009, 15 more plants died, and 22 plants died until the last measurement on 19 August 2009 (data not shown).

Table 10.2: Results of mixed-effects model analysis of initial rosette diameter (covariate), clipping treatment (fixed), 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 six phenotypic traits of *Campanula barbata* in the common garden. See Methods for details on GLMMs. Chi^2 values and their significancies were obtained from model comparisons using likelihood ratio. *%VC*—Variance components were obtained from analyses with all factors treated as random effects. df—degrees of freedom, residual df varies per trait due to mortality and due to flowering traits being recorded in flowering plants only.

		Rosette diameter			Number of leaves			Plant height		
	df	Chi^2		*%VC*	Chi^2		*%VC*	Chi^2		*%VC*
Clipping treatment	1	15.0	***	4.8	11.0	***	3.6	1.8		0.8
Region	1	1.5		0.0	2.5		0.0	41.1	***	15.6
Population (Region)	1	60.4	***	12.4	32.3	***	6.7	100.9	***	20.0
Seed family (Population)	1	14.9	***	9.3	3.7	***	16.1	13.2	***	6.9
Clipping×Region	1	1.0		0.0	0.9		0.0	0.2		0.0
Clipping×Pop (Region)	1	0.0		0.9	0.0		0.0	0.0		0.0
Clipping×Seed family (Population)	1	0.0		0.0	0.4		2.6	0.0		0.0
Residuals	§			72.6			71.0			56.7
		Number of inflorescences			Number of flowers			Biomass		
	df	Chi^2		*%VC*	Chi^2		*%VC*	Chi^2		*%VC*
Clipping treatment	1	10.3	**	2.9	6.2	*	8.2	24.0	***	8.8
Region	1	11.1	***	3.3	0.1		0.0	0.0		0.0
Population (Region)	1	9.8	**	3.2	16.3	***	8.0	28.2	***	8.6
Seed family (Population)	1	8.2	**	7.0	1.9		8.5	2.9		3.8
Clipping×Region	1	0.0		0.0	1.5		1.1	2.8		0.7
Clipping×Pop (Region)	1	0.0		0.0	0.0		0.0	0.0		0.0
Clipping×Seed family (Population)	1	0.0		0.0	0.0		0.0	0.0		0.3
Residuals	§			83.6			74.1			77.7

The residual degrees of freedom is 587, 588 and 566 for rosette diameter, number of leaves and plant height, respectively.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

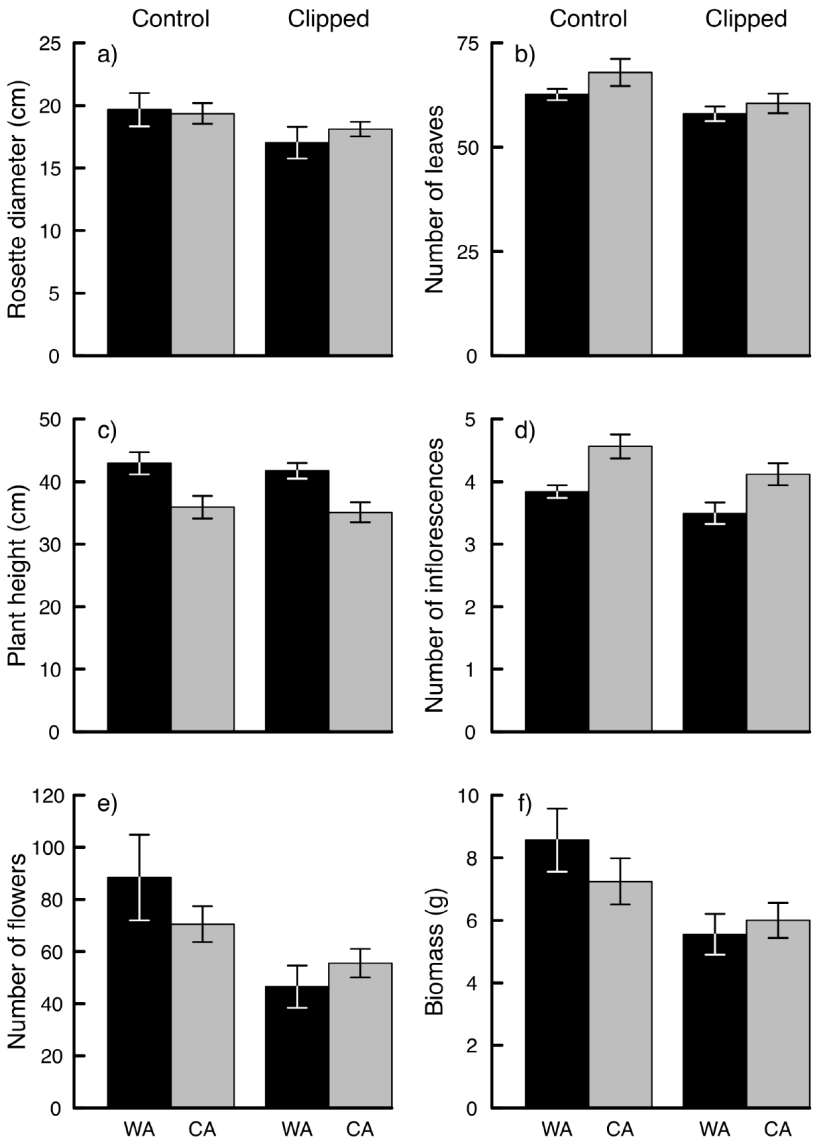

Figure 10.2: Mean values of morphological traits of *Campanula barbata* for the two regions (Western Alps – WA; Central Alps – CA) and treatments (control versus clipped plants) in the common garden. a) Rosette diameter; b) Number of leaves; c) Plant height; d) Number of inflorescences; e) Number of flowers; f) Biomass. Means (SE) are based on population means, which in turn are based on seed family means. WA: n= 4; CA: n=11.

Local adaptation

Correlations between trait values measured in the common garden and altitude of origin were significant and negative for number of leaves, plant height and above-ground biomass (Table 10.4). When the region effect was removed, these correlations became stronger. Plant traits never correlated with precipitation, whereas number of leaves correlated strongly and positively with minimum temperature and plant height correlated positively with minimum, mean and maximum temperature.

Table 10.3: Results of mixed-effects model analysis of initial rosette diameter (covariate), clipping treatment (fixed), 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 six phenotypic traits of *Campanula barbata* in the common garden. See Methods for details on GLMMs. Chi^2 values and their significancies were obtained from model comparisons using likelihood ratio. df—degrees of freedom.

	df	Rosette diameter Chi^2	Number of leaves Chi^2	Plant height Chi^2	Number of inflorescences Chi^2	Number of flowers Chi^2	Above-ground biomass Chi^2
Distance to common garden	1	8.3**	8.4**	14.8***	9.0**	3.2	1.4
Clipping treatment	1	15.2***	11.1***	1.8	10.4**	6.6**	24.1***
Region	1	8.7**	5.2*	41.4***	2.0	6.7**	5.2*
Population (Region)	1	47.4***	23.9***	87.7***	9.9**	10.4**	23.8***
Seed family (Population)	1	14.8***	38.3***	13.3***	8.1**	1.9	2.7
Clipping×Region	1	1.0	0.9	0.2	0.0	1.7	2.8
Clipping×Pop (Region)	1	0.0	0.0	0.0	0.0	0.0	0.0
Clipping×Seed family (Population)	1	0.0	0.4	0.0	0.0	0.0	0.0

Residual degrees of freedom, see Table 10.2
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 10.4: Correlations between measured plant traits in the common garden and various environmental factors of origin in *Campanula barbata*. Shown are Pearson's correlation coefficient with asterisks indicating *P*-values.

	Alt_1	Alt_2	Prec	T_{min}	T_{mean}	T_{max}
Rosette diameter	-0.35	-0.35	0.01	0.22	0.16	0.10
Number of leaves	-0.60*	-0.63*	-0.23	0.64**	0.48	0.32
Plant height	-0.57*	-0.66**	-0.11	0.53*	0.53*	0.53*
Number of inflorescences	-0.31	-0.37	-0.04	0.28	0.11	-0.06
Number of flowers	-0.37	-0.39	0.10	0.20	0.30	0.32
Above-ground biomass	-0.53*	-0.55*	0.10	0.39	0.34	0.30

Alt_1—Altitude of population origin
Alt_2—Altitude of population origin after region effect is subtracted (see Methods)
Prec—Precipitation
T_{min}—Minimum average monthly temperature
T_{mean}—Yearly average temperature
T_{max}—Maximum average monthly temperature
* $P < 0.05$; ** $P < 0.01$

Discussion

Glacial history related phenotypic differentiation

The detected regional differentiation in the reproductive traits of plant height and number of inflorescences (Table 10.2, Fig. 10.2c–d) is in line with the phylogeographic structure but could be due to distance-related effects. Including distance to the common garden in the model led to significant differences between the two regions for all traits except number of inflorescences (Table 10.3). The latter results are stronger support for the hypothesis that glacial history-related molecular differentiation is mirrored by phenotypic differentiation, since distance-related gradual change in environmental conditions was removed. The molecular

break line running from Lucerne to Lugano (Thiel-Egenter et al. 2011) is presumed to be related to glacial history in such a way that populations from either side of the break line belong to lineages which survived the last or multiple glaciations in separate refugia on the fringes of the Alps or on nunataks within the Alps (Schönswetter et al. 2005; Stehlik 2000). Our results suggest that this long-term isolation caused not only neutral molecular differentiation, but also phenotypic differentiation in most traits. A similar conclusion was drawn in studies with other species. Both *Campanula thyrsoides* (Scheepens et al., unpublished) and *Geum reptans* (Frei et al., unpublished) showed phenotypic differentiation among four and three phylogeographic lineages across the European Alps, respectively.

Clipping of leaves sets back the absolute growth rate and removes valuable biomass. Clipping could therefore be hypothesised to have long-lasting negative effects on both vegetative traits and reproductive output (Strauss and Agrawal 1999). In line with this prediction, our results showed a decrease in rosette diameter, number of leaves, inflorescences and, particularly, flowers, as well as strongly decreased biomass. Plant height, i.e. height of the inflorescences, was not affected, which is evidence that plant height is not affected by the environment but is genetically determined. Since we found no interaction with region, our results suggest that glacial history did not affect the response of plants to a removal of biomass.

Populations explained more variation in plant height than region did. Among populations plant height ranged from 25–47 cm for unclipped plants. As the correlations of plant height with environmental variables showed, part of this population differentiation could be the result of adaptation to the population-specific environment (Clausen et al. 1948; Joshi et al. 2001). This indicates that the environmental heterogeneity of the Alps may be a stronger force for differentiation than random or selective effects due to glacial history.

The fact that seed families differed significantly in most phenotypic traits indicates that these traits have a strong heritable component and that populations harbour considerable variation. This could benefit the survival of populations through, for instance, climate change by adapting to changing environmental conditions (Davis and Shaw 2001; Jump and Peñuelas 2005; Visser 2008). Additionally, since several phenotypic traits correlated with altitude and other environmental factors at population origin (Table 10.4), *C. barbata* may be able to persist through receiving adaptive genetic material by migration or gene flow from populations at lower elevations (Davis and Shaw 2001). Nevertheless, the occurrence of *C. barbata* is common which allows speculating that upward migration to track climate belts would not be hampered (Grabherr et al. 1994; Walther et al. 2005; Parolo and Rossi 2008).

The absence of any significant interactions suggests that neither the two phylogeographic lineages nor populations nor seed families evolved different responses to herbivory. Though the similar response of plants to clipping may be the result of similar phenotypic plastic responses, it rather suggest that the response is passive (i.e. apparent plasticity, Wright et al. 2002; Weiner 2004). Data on herbivory are lacking from these two regions, which precludes sound speculation on expectations. The fact that the Western Alps are subject to sheep herding rather than cattle herding, as is dominant in the Central Alps, could be investigated for its possible effects on herbivory response in *C. barbata*.

Distance to the common garden

Comparing the results of models with and without distance to the common garden indicated that the observed regional differentiation in number of inflorescences was not due to phylogeographic lineage but due to gradual change across the sampled Alpine range. Distance to the common garden significantly explained variation in number of inflorescences, which could be either an effect of isolation by distance or an effect of adaptation to environmental conditions which change linearly with distance, but it is difficult to pinpoint which factor or group of factors could be responsible for adaptation. Variation in rosette diameter, number of leaves and plant height was partly explained by distance to the common garden, but also retained differentiation due to phylogeographic region which strengthens the claim that glacial history affected these traits (Table 10.3).

Local adaptation

Local adaptation to specific environmental conditions is common in plants and has frequently been investigated along clines (e.g. Del Pozo et al. 2002; Olsson and Ågren 2002; Santamaría et al. 2003; Ramírez-Valiente et al. 2009). The altitudinal range of investigated populations in our study is 1797–2440 m, and the difference of 643 m would theoretically account for 3.5 K temperature difference (Ozenda 1988), all else being equal. The difference in the highest and lowest extrapolated mean temperatures was actually 4.4 K. During the winter the maximum difference was only 2.9 K, but during summer sites differed maximal 7.2 K. These temperature differences are large enough for specific adaptations to evolve. Three traits – number of leaves, plant height and above-ground biomass – indeed correlated with altitude, whether without or with the effect of phylogeographic region removed (Table 10.4). Temperature explained number of leaves and plant height but not biomass. Although the range in precipitation was large (1097 mm), phenotypic traits did not correlate with precipitation. Therefore, other factors that change with altitude should account for the decrease in biomass, such as decrease in soil nutrient status with increasing altitude due to wash out to lower altitudes (Körner 2003).

Without adjusting for distance-related effects, our measurements showed that inflorescence height was larger but number of inflorescences was lower in the Western Alps compared to the Central Alps (Fig. 10.2c–d), which could imply a trade-off. Direct Pearson's correlations between plant height and number of inflorescences were significant and positive (r=0.17, P<0.0001) as were correlations of number of flowers with plant height (r=0.42, P<0.0001) and with number of inflorescences (r=0.53, P<0.0001). Thus, taller plants have more inflorescences and more flowers, but since allocation patterns can change with the size of plants (Weiner 2004), a trade-off between plant height and number of inflorescences could still be present.

The significant negative correlation of plant height with altitude (Table 10.4) therefore indicates that plants become smaller and bear less inflorescences and flowers with increasing elevation (although number of inflorescences and flowers were not themselves correlating with altitude). Similar negative relationships of altitude with final height were found in a common garden experiments with *Senecio inaequidens* originating from two contrasting altitudinal transects from northern Belgium and the French Pyrenees (Monty and Mahy 2009). The grass *Festuca eskia* showed similar negative correlations of altitude with plant height (Gonzalo-Turpin and Hazard 2009).

An explanation for this correlation could be that lower stature allows the plant to decouple its own climate from the ambient conditions, allowing the plant to heat up more efficiently in a colder environment (Körner 2003). This is favourable since the growing season is short and temperatures low at high elevations. At lower elevations, with a longer growing season and higher temperatures, taller plants are favoured as this may benefit seed dispersal (Tackenberg et al. 2003) besides cooling of the leaves (Körner 2003). Smaller plants at high elevation may also be an adaptation to harsher conditions (Galen et al. 1991). Maternal effects could be an alternative explanation for the correlation between plant height and altitude, since experimental plants were derived from seeds directly sampled from populations of origin and therefore could be influenced by the maternal environment. For instance, the nutritional status of populations could have influenced seed quality and thereby confer non-genetic phenotypic variability to the offspring. Maternal effects have their strongest influence on seedling traits and usually diminish over time (Ouborg et al. 1991; Ouborg and Van Treuren 1995). However, maternal effects can also be transmitted to later stages (Roach and Wulff 1987), especially when unequal intraspecific competition is present (Schmid and Dolt 1994). The rosette diameter at the start of the experiment can be used to remove potential maternal effects on traits at later life stages, although it may also remove part of the effects due to genetic differentiation. When performing correlations of altitude with plant height and number of inflorescences while the effect of rosette diameter at the start of the experiment was statistically removed, plant height remained significant (r=-0.52, P<0.05) but number of inflorescences lost its significancy (r=-0.08, P=0.69).

This suggests that the observed correlation of plant height with altitude is due to genetic differentiation, whereas evidence for number of inflorescences is inconclusive as the results may be due to either genetic differentiation or maternal effects. The fact that plant height is not affected by rosette size and therefore probably not by nutritive status is in line with the fact that plant height did not change under the clipping treatment (Fig. 10.2c). Besides, if taller and more inflorescences would be the result of better nutritive status as seed, then this would probably be not related to altitude since intraspecific variation in seed weight was shown to be unrelated with altitude in four different species (Pluess et al. 2005).
We did not perform any corrections for multiple testing, but based on a Bernoulli process (Moran 2003) the probability that these results would appear by chance ranged from $1.8\times10^{-6} < P < 0.03$ for the different sets of tests (each time six traits taken together in either mixed-effects models or Pearson's correlations).

Effects of glacial history

Our analyses indicated a role for glacial history on phenotypic differentiation. This differentiation came into existence either through neutral processes or through adaptation. If molecular marker data is available from the populations, Q_{ST}–F_{ST} analyses could be applied to determine whether adaptation had a role in differentiation (Ramírez-Valiente et al. 2009). This test does not, however, tell us when this differentiation took place, which could be either during glacial survival, during recolonisation or during recent times. The possibility that the differentiation is due to current adaptation seems unlikely given the long glacial history of Alpine plants, but is not impossible since major biogeographic regions are related with regional environmental conditions, which could even be related to topography. For instance, outer mountain chains usually receive much more precipitation than the relatively dry inner-alpine valleys (Ozenda 1988). Also, geomorphological conditions, such as silicolous versus calcicolous plants, can play an important role not only in the current distribution of species (Braun-Blanquet and Jenny 1926; Kinzel 1983) but also in their patterns of postglacial recolonisation and molecular marker differentiation (Alvarez et al. 2009).
Based on the widespread and common occurrence of *Campanula barbata* in subalpine and alpine meadows, one could hypothesise that connectedness among populations would be high and that signals of glacial history would be diluted. However, the observed phylogeographic differentiation is strongly present, comparable with species with much stronger isolation, such as *Geum reptans* (Frei et al., unpublished) and *Campanula thyrsoides* (Scheepens et al., unpublished). This raises the question whether topography of the Alpine landscape could play a role in restricting current gene flow.

Conclusions

This study showed that glacial history affected phenotypic differentiation in *C. barbata*. The two studied phylogeographic lineages differed with respect to most investigated traits. It remains uncertain how much of the phylogeographic differentiation is the result of adaptation and how much of neutral genetic drift. In contrast, correlations between trait values and environmental factors suggested that number of leaves, plant height and biomass are locally adapted to current conditions. Both differentiation due to glacial history and current local adaptation indicate that within-species genetic diversity is present at regional and local scales. The widespread occurrence of *C. barbata* and its substantial seed family differentiation may prove favourable for survival of populations through climate change.

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Chapter 11

Regional differences in growth, reproduction and leaf morphology mirror phylogeography of a widespread Alpine plant

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Regional differences in growth, reproduction and leaf morphology mirror phylogeography of a widespread Alpine plant

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Abstract

Glacial history has affected the phylogeographic structure of numerous Alpine plants, but its impact on phenotypic differentiation has rarely been studied. Here, we aimed further than most other studies and asked, whether phylogeographic structure is also mirrored by regional phenotypic differentiation of the widespread Alpine plant *Geum reptans* L. We combined a molecular study with a common garden experiment and investigated genets from 16 populations of *G. reptans* sampled across the European Alps. Using neutral molecular markers (RAPDs) and a Bayesian cluster analysis, we analysed genetic differentiation and phylogeographic structure. In the common garden, differentiation in phenotypic traits related to growth, reproduction and leaf morphology, as well as the response of plants to competition, were measured. The molecular analysis revealed a partitioning of the populations into three genetic groups, which represent a pronounced phylogeographic structure. Moreover, the regional molecular structure is well in line with regional phenotypic differentiation and regional variation in growth response to competition inferred from mixed-effects modelling. In order to test the hypothesis whether selection played a role in the observed quantitative trait differentiation (Q_{ST}), we compared Q_{ST} with neutral molecular differentiation (G_{ST}). Q_{ST} was different from G_{ST} for 10 out of 11 traits, indicating that selection contributed to phenotypic differentiation. Significant negative correlations between biomass and precipitation data of population origin are an additional indication of adaptation. Synthesis. The current study compared regional molecular and phenotypic differentiation among populations of a widespread plant in the historical context of drastic range changes, which occurred during the glaciations in the Alps. Since the phylogeographic structure is reflected by strong phenotypic differentiation, we conclude that historical forces affected both genotypes and phenotypes. Our results suggest that, in addition to genetic drift and limited gene flow during survival in glacial refugia and postglacial remigration, part of the observed phenotypic differentiation resulted from adaptation to current climatic conditions. Interpreted in the context of Quaternary climatic oscillations, our findings are relevant with respect to the future adaptive potential and migration patterns of Alpine plants due to climate change.

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

Introduction

The understanding of the historical background of regional differences in plant populations has increased substantially since the development of molecular methods (Sunnucks 2000). In the last decade, the impact on the genetic make-up of species due to cyclical and drastic range changes during climatic oscillations and glaciations in the Quaternary has received particular attention (Hewitt 1996, 2000; Taberlet et al. 1998). In the European Alps, phylogeographic studies demonstrated that the effect of population differentiation, which occurred within species that survived in glacial refugia outside the Alps, is still detectable in regional molecular differentiation of 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 for neutral molecular differentiation in plants (Till-Bottraud and 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 among Alpine plant populations (Schönswetter et al. 2005). Molecular phylogeographic differentiation is detectable among present-day populations of Alpine plants for two reasons. One possible explanation for a phylogeographic structure is that the period of time since the last glaciation (c. 10,000 years ago) was too short for different phylogeographic lineages to mix. It could also be that current gene flow is too weak to mask the historical effects completely, since it is limited by dispersal barriers, such as deep valleys or high mountain chains characterising the present landscape of the Alps (Körner 2003).

One can hypothesise that glacial history also had an impact on phenotypic differentiation of Alpine plants. In contrast to random genetic drift leading to neutral differentiation (Nei, Maruyama and Chakraborty 1975), directional selection leads to adaptive differentiation maintaining or enhancing fitness in different environments (Kawecki and Ebert 2004). Adaptive regional differentiation in phenotypic traits is well known in widespread plant species (Joshi et al. 2001; Olsson and Ågren 2002; Becker et al. 2006), and can be expected in Alpine plants due to two main reasons. First of all, Alpine plants may have experienced historical selection caused by local conditions in glacial refugia outside the Alps (Hewitt 1996). Secondly, selection by current environmental conditions is also likely, by either regional climatic differences over the Alpine belt or locally by the distinct spatial and temporal heterogeneity of Alpine habitats (Till-Bottraud and Gaudeul 2002). Therefore, neutral processes during glaciations and post-glacial recolonisation, as well as historical or current adaptive processes, may have affected phenotypic differentiation in widespread Alpine plants.

While selection leading to adaptation is a long-term process that takes place over many generations, phenotypic plasticity allows plants to adjust more rapidly to environmental variation and at a more fine-grained scale (Sultan 2000). Phenotypic plasticity is a complementary mechanism for phenotypic differentiation and is considered a genetic trait in itself (Schlichting and Smith 2002). Regional variation in adaptive plasticity has already been observed in lowland plants (e.g. Berg, Becker and Matthies 2005) and might be particularly pronounced in Alpine plants, since their habitats are subjected to high climatic variation (Gonzalo-Turpin and Hazard 2009).

Most phylogeographic studies which have investigated the impact of glacial history on within-species differentiation are restricted to neutral molecular markers (for a review see Schönswetter et al. 2005). Our study is among the first that compares molecular differentiation directly to phenotypic differentiation in the phylogeographic context of Alpine glaciations (but see Lagercrantz and Ryman 1990). Here, we investigated whether the phylogeographic structure inferred from putatively neutral molecular markers (RAPDs) is mirrored by regional differentiation in phenotypic traits related to growth, reproduction and leaf morphology. We analysed genets of the widespread Alpine plant *Geum reptans*. The genets were sampled from 16 populations across the species' distribution in the Alps (Fig. 11.1) and used in a common garden experiment as well as in a molecular analysis. Common garden experiments are a powerful and frequently used tool to help reveal genetic differentiation in phenotypic traits among regions and populations (e.g. Olsson and Ågren

2002). By including an environmental treatment (e.g. competition) in a common garden experiment, phenotypic plasticity can be measured and investigated as the response to this treatment (Pluess and Stöcklin 2005). We therefore grew clonal progeny of *G. reptans* in the common garden with and without competition from the alpine grass species *Poa alpina* L. However, common garden experiments are not suitable for distinguishing between neutral evolutionary forces such as drift and adaptive processes affecting phenotypes (Kawecki and Ebert 2004), but other methods may suggest adaptation such as correlations between traits measured in a common garden and environmental variables at the original sites of populations (Linhart and Grant 1996) or Q_{ST}-F_{ST} comparisons (Merilä and Crnokrak 2001). Neutral molecular differentiation (F_{ST}) can be used as a measure for background genetic drift and any deviation in quantitative trait differentiation (Q_{ST}) from F_{ST} indicates selection (Spitze 1993). In our study, we addressed the following questions: (1) Is the phylogeographic structure of *G. reptans* inferred from putatively neutral molecular markers in line with regional phenotypic differentiation observed in a common garden? (2) If present, is any 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 also affected phenotypic differentiation? (4) Can regional variation be detected in the response of plants to competition?

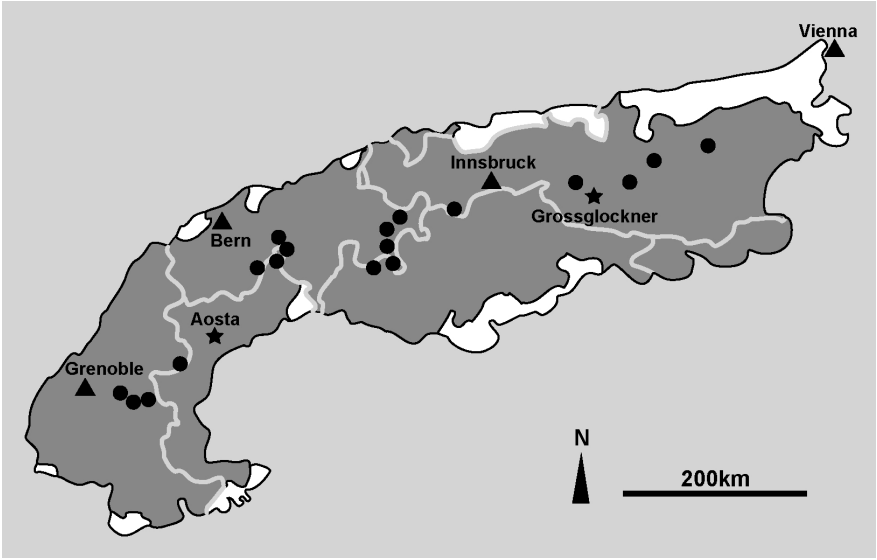

Figure 11.1: Distribution and locations (dots) of populations of *Geum reptans* sampled across the European Alps. Darkgrey areas show where the species is occurring actually and white areas where it is absent. Light grey lines represent borders of the countries. Aosta, Aosta valley; Grossglockner, Grossglockner mountains. Map modified from Aeschimann et al. (2004).

Methods

Study species

Geum reptans has a distribution encompassing the entire European Alps, and eastwards 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 silicious bedrock (Aeschimann et al. 2004). After glacier retreat, *G. reptans* is one of the first pioneers on the virgin soils and persists until competition with other species becomes too strong (Weppler and Stöcklin 2005). Plants of *G. reptans* can reproduce sexually, by producing 1–5 flowering stems with a terminal flower head, or clonally, by forming new rosettes at the tip of stolons (Pluess and Stöcklin 2005).

Common garden experiment

In the late summer of 2007, plant material was collected from 16 populations at different sites (Fig. 11.1; see also Appendix Table 11.7). To obtain a representative coverage of its distribution in the European Alps, the sampling area spanned all biogeographic regions that are assumed to reflect the spatial genetic structures within Alpine plant species (Schönswetter et al. 2005). In each population, we sampled a minimum of four stolons with rosettes (ramets) from 20 genets. The space between sampled genets was at least 5 meters to minimise the risk of resampling the same genotype. Rosettes were kept in plastic bags in a fridge until planted in separate pots of 10 × 10 × 10 cm^3. Pots were filled with a 1:1 mixture of river gravel and potting soil, and were kept on tables in a greenhouse in Basel and randomised weekly. We applied the organic insecticide Traunem (Andermatt Biocontrol AG, Grossdietwil, Switzerland) twice to the plants to control infestations of Sciaridae. Four weeks before transplantation, the plants were transferred outside the greenhouse for acclimatisation.

On the 19th of May 2008, the plants were transferred to the common garden, a fenced area of 3 × 10 m^2 in the Central Alps in Davos (1532 m a.s.l.). From each of the 16 populations, we planted four ramets of 8–14 genets in the garden (n = 592 plants). We used a randomised block design, ensuring that each sampled population was present with an equal number of individuals in each of the four blocks. Two out of four ramets of each genet were surrounded by seedlings of *Poa alpina* (seeds originated from the Austrian Alps, Otto Hauenstein Samen, Landquart, Switzerland). The treatment with *Poa alpina* was used to simulate interspecific competition and to measure the growth response of plants to competition. Plants did not need watering, but the garden was regularly weeded and the competitor, *Poa alpina*, was clipped four times to prevent too strong above-ground dominance.

The initial plant diameter was measured immediately after transplantation. Traits related to growth (number of leaves), reproduction (number of reproducing individuals, number of flowers and stolons) and leaf morphology (length, width and number of leaflets of longest rosette leaf and specific leaf area) were measured after two growing seasons in June 2009. On the 3rd of July 2009, plants were harvested; 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 the relative strength of the phenotypic plasticity of plants in response to competition, we followed the method used by Snaydon (1991). First, for each genet, we calculated the difference in the average log (biomass) of ramets with and without competition. Second, to obtain a relative measure for competitive ability, we subtracted the previously calculated difference from one. Higher relative competitive ability of a genet indicated stronger phenotypic plasticity. As a measure for the relative importance of sexual versus clonal reproduction, we calculated the proportion of reproductive meristems (sum of flowers and stolons) that consisted of stolons. As an indicator of leaf shape, we calculated the ratio of leaf length to width. To estimate the number of leaflets, all secondary veins branching from the leaf midrib were counted and as a measure of leaf dissection, the number of leaflets was divided by leaf length. Specific leaf area (SLA) was measured on a subset of plants which were not surrounded by the competitor (n = 125). Five circular leaf corings with an area of 44 mm^2 were taken from different rosette leaves of one individual and were dried at 60°C for 48 h. All leaf corings of one individual were weighed together. SLA was then calculated as the fresh leaf area divided by the dry weight of the corings according to Cornelissen et al. (2003).

RAPD fingerprinting

Leaf material of eight genets from each of the populations used in the common garden experiment (n = 128) was analysed with RAPD fingerprinting (Williams et al. 1990). DNA extraction from dried leaf material and measurement of DNA concentration was 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[GTGGGC TGAC], OPP17[TGACCCGCCT], OPP8[ACATCGCCCA] and OPP9[GTGGTCCGCA]. The RAPD-PCR was done with self-dissolving Illustra^{TM} puReTaq Ready-To-Go PCR Beads (GE Healthcare,

Buckinghamshire, UK). The PCR volume (25 μL) contained 10 mM Tris-HCl buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl and 2.5 U polymerase (with all mentioned substances contained in the beads). In addition, 6 ng of DNA, 25 pmol primer and ddH_2O were added to each PCR bead. PCRs were always run in the same machine (Mastercycler gradient, Eppendorf, Hamburg, Germany), with the following cycle conditions: 120 s at 94°C for initial denaturation, followed by 34 cycles of 92°C for 30 s, 36°C for 30 s and 72°C for 90 s and a final extension step of 72°C for 300 s. PCR products were separated on 2% agarose gels in 1× Tris-Borate-EDTA buffer with 100 bp DNA ladders as size standard. Gels were stained with ethidium bromide.

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

Molecular analyses

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

To investigate the genetic structure, genets were assigned to genetic clusters using a model-based Bayesian cluster analysis. We used the algorithm for dominant markers (Falush, Stephens and Pritchard 2007) and a standard admixture model with independent allele frequencies (Pritchard, Stephens and Donelly 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 K (number of clusters) ranging from 1–10. The ad-hoc statistic ΔK was used to identify the most likely number of clusters within the data set (Evanno, Regnaut and Goudet 2005). Molecular data of four genets from each of two additional populations (STAU and TTAU, see Appendix Table 11.7) from the Eastern Alps were included in the cluster analysis to check for the continuity of the most eastern phylogenetic group (Fig. 11.2). The two latter populations were not included in the common garden experiment, but leaf material for the RAPD analysis was provided by the IntraBioDiv Consortium (Gugerli et al. 2008).

To test for isolation-by-distance (Wright 1946), we correlated pairwise genetic distances (Nei 1978) with the geographic distances of populations and performed Mantel tests for populations of all phylogeographic regions and the Central Alpine region separately in GENALEX version 6.0 (Peakall and Smouse 2006). The assignment of populations to regions is described in detail below. Significance level of the Mantel correlation coefficients R was obtained after performing 1,000 permutations. The partitioning of molecular variance among regions, populations within regions and genets within populations was determined using an AMOVA (Excoffier, Smouse and Quattro 1992). Fixation indices were computed and tested by 1,000 permutations for each level of the genetic structure: Φ_{RT} for variation among regions, Φ_{PR} for variation among 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 of *G. reptans* to the three phylogeographic regions as inferred from the Bayesian cluster analysis of molecular data, including Western, Central and Eastern Alps (Fig. 11.2a). 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 with the highest likelihood for the posterior distribution (Ln P) of the data out of 20 runs. We used three regions because the assignment probabilities of populations for four regions ($K = 4$; Fig. 11.2b) were too weak for a clear assignment for 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 with STRUCTURE was repeated with only Central Alpine populations (results not shown).

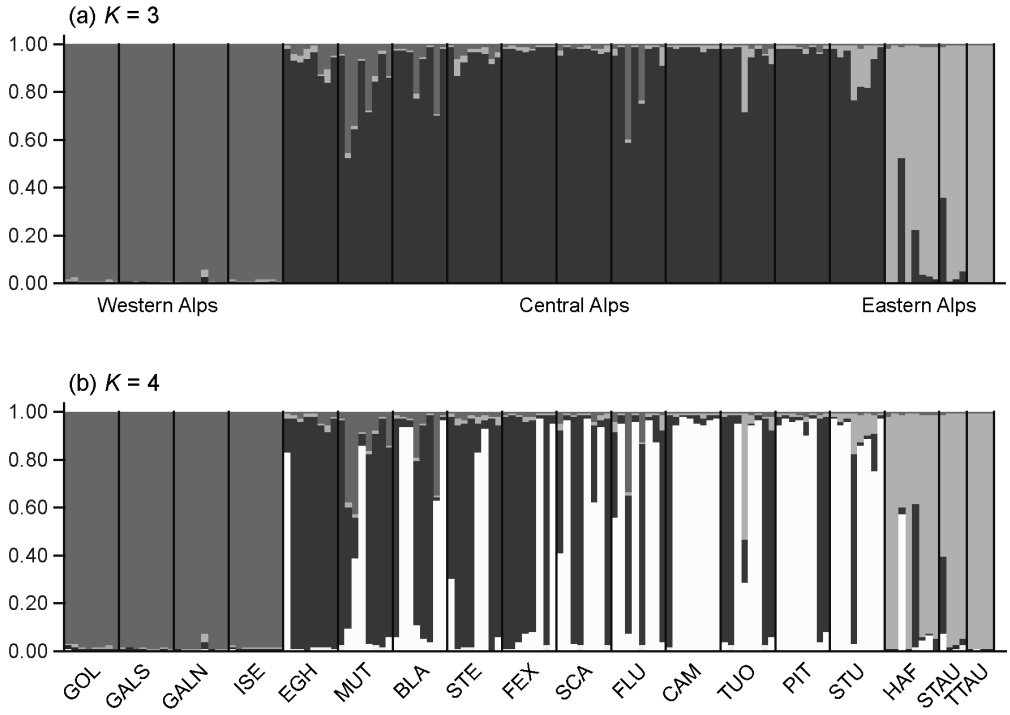

Figure 11.2: Molecular differentiation of genets from populations of *Geum reptans* sampled across the Alps for (a) $K = 3$ clusters and for (b) $K = 4$ clusters inferred from a Bayesian cluster analysis with the program STRUCTURE. The different clusters (phylogeographic regions) are represented by different shades of grey. Genets are grouped to populations which are aligned from Western (left) to Eastern Alps (right). Bars indicate the assignment probability Q of the genets to be a member of one of the clusters. Shown is the simulation run with the highest likelihood for the posterior distribution (Ln P) of the data out of 20 runs for each K.

Linear modelling

We used mixed-effects modelling to investigate genetic (phylogeographic region, population and genet) and environmental (competition) effects on traits that were measured in the common garden experiment. To analyse the frequency of reproduction with a binomial error distribution, we fitted generalized linear mixed models (GLMM) with a *logit* link function. For all the continuous variables with normal error distributions we fitted linear mixed models (LMM). In both models, we used restricted maximum likelihood (REML), because they are better used with unbalanced data sets (unequal number of populations per region) than classical ANOVAs (McCulloch and Searle 2001). The mixed-effects models were calculated by means of the *lmer* function in the R package LME4 (Bates and Maechler 2009). The most complex model included the initial plant diameter as a covariate, 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 used as a random effect to account for potential 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 the random effects by calculating their variances and tested the significance of the random effects using likelihood ratio tests following the method used by Pinheiro and Bates (2000). We checked all model assumptions using diagnostic plots constructed with the R packages LATTICE (Sarkar 2009) and ASUR (Fabbro 2007). Biomass, number of leaves, number of flowers and number of stolons were transformed using the natural logarithm to circumvent violations of model assumptions. Tukey HSD post-hoc tests were used to test the differences between the means of all region pairs.

To answer the question of how much variation can be attributed to the 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 *VarCorr* function in the R package LME4 (Bates and Maechler 2009) and the corresponding variance components V were calculated based on the methods used by Crawley (2007).
To analyse regional variation in phenotypic plasticity, we fitted linear models with the relative competitive ability in terms of the response in growth (i.e. leaf and root mass). The effects of *Region*, *Population* and *Genet* were nested in each other and were tested with ANOVAs. Another ANOVA was run to test for regional differences in genetic diversity H_e. Contrast tests with the function mancontr in the R package ASUR (Fabbro 2007) were used to test the differences between the means among 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

In order to evaluate if any phenotypic differentiation is the result of selection, we compared the quantitative trait differentiation (Q_{ST}) of all phenotypic traits measured in the common garden experiment with a neutral molecular differentiation index (G_{ST}). When a trait differentiated in a neutral manner, Q_{ST} should theoretically equal G_{ST}. In contrast, a trait is assumed to have been under selection, when Q_{ST} differs from G_{ST}, i.e. unifying ($Q_{ST} < G_{ST}$) or diversifying ($Q_{ST} > G_{ST}$) selection (Merilä and Crnokrak 2001). We calculated Q_{ST} according 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 with jackknifing over populations (O'Hara and Merilä 2005). To test whether Q_{ST} was significantly different from G_{ST}, we checked whether the 95% confidence intervals of means overlapped with the G_{ST} value. All calculations were performed using R version 2.10.0 (R Development Core Team 2009).

Correlation analyses

To test whether trait differentiation is related to climate, we performed Pearson's correlation analyses in R version 2.10.0 (R Development Core Team 2009) between all phenotypic traits and climatic data of population origin. Climatic data were obtained from the WorldClim database (http://www.worldclim.org). WorldClim is a set of global climate grids with a spatial resolution of 150 arc-seconds based on monthly climatic data from weather stations recorded during the years 1950–2000 (Hijmans et al. 2005). Of the WorldClim data points surrounding each population location, we selected the one that had the least altitudinal difference with the population location. The temperature data were corrected for the difference in altitude by adding or subtracting 0.0055°C per meter (Ozenda 1988). We calculated annual total precipitation, annual mean temperature, summer temperature (mean for the months June–August), minimum and maximum temperature based on the monthly climatic data and averaged annual data over the last fifty years. Since variables other than temperature can change with elevation, we analysed the correlation between altitude of population origin and all phenotypic traits. By correlating climatic data and altitude of population origin with the residuals obtained from ANOVAs with *Region*, we could make a stronger case for adaptation, since 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 was scored in all investigated genets. Only two out of 53 markers were monomorphic. Genetic diversity in all studied populations of *G. reptans* was $H_E = 0.14 \pm 0.04$ (mean ± SD) with a range of 0.07–0.21, and was significantly different among 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)

in the West Alpine populations (Fig. 11.3a). The average genetic differentiation among populations was $G_{ST} = 0.395$ (95% CI 0.388–0.399). Bayesian cluster analysis using molecular data resulted in a distinct phylogeographic structure with three genetic clusters ($K = 3$) having the best ad-hoc statistical fit ΔK (see Appendix Fig. 11.7). When setting $K = 3$, populations were grouped into a West, Central and East Alpine group (Fig. 11.2a). For $K = 4$, the distinct Central Alpine group splits into two new groups, dividing the Central Alpine populations into a western and an eastern part, but with a large admixture zone and no clear geographic boundary (Fig. 11.2b). Pairwise genetic distances varied from 0.02–0.27 and were significantly correlated with geographic distances when all populations were included in the Mantel test ($R = 0.80$, $P < 0.01$), as well as when Central Alpine populations were analysed separately ($R = 0.58$, $P < 0.01$; Fig. 11.4). The AMOVA revealed that the molecular differentiation among the three phylogeographic regions explains a large part (36%) of the total molecular variation, while differentiation among populations within regions accounts for 10% (Table 11.1).

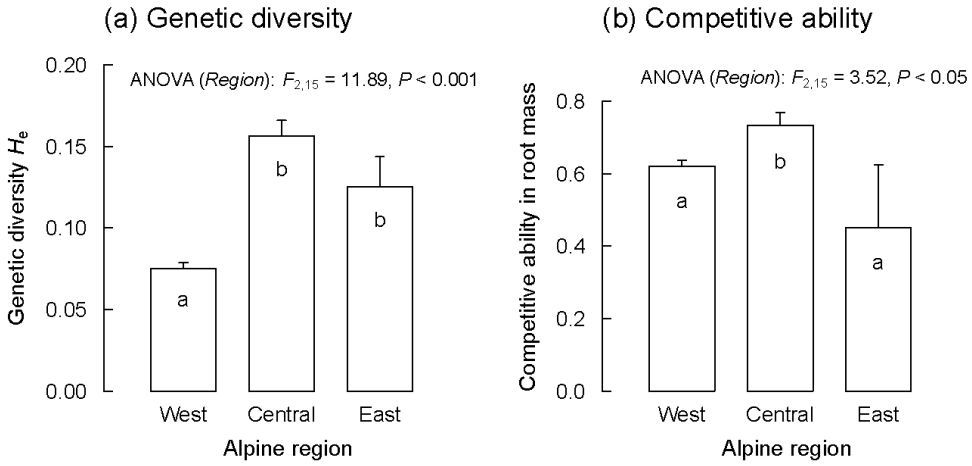

Figure 11.3: Regional differences in (a) genetic diversity H_E and (b) competitive ability in root mass of genets derived from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps. Genets were grown with and without competition from the grass *Poa alpina* in a common garden to assess phenotypic plasticity in competitive ability (sensu Snaydon 1991). Bars show means + SE. *F*- and *P*-values are from ANOVAs. Significance of differences among regions was obtained with contrast tests at the $\alpha = 0.05$ level and is represented by letters.

Table 11.1: Analysis of molecular variance (AMOVA) with molecular data of genets derived from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps

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

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

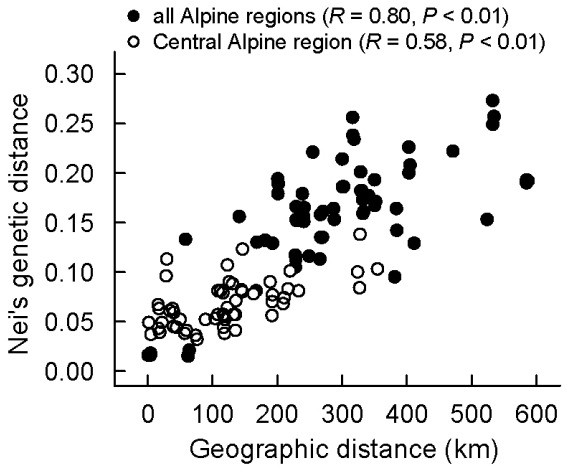

Figure 11.4: Correlation of pairwise genetic with geographic distances of 16 populations of *Geum reptans* from three phylogeographic regions in the Alps. Shown in the graph are correlation coefficients R and P-values from Mantel tests for all populations (filled and open dots) and for Central Alpine populations (open dots).

Phenotypic differentiation

Significant regional differentiation was present in all traits related to growth, reproduction and leaf morphology (Tables 11.2 and 11.3). Plants originating from the West and East Alpine regions were larger, with a greater total biomass and leaf mass and also more rosette leaves than Central Alpine plants (Fig. 11.5a). Allocation of biomass to the roots was highest in plants from the west and decreased towards the Eastern Alps (Fig. 11.5b). In contrast, Central Alpine plants had more flowers and stolons than plants from the other two regions (Fig. 11.5c). Clonality decreased (Fig. 11.5d) as reproduction increased in frequency from west to east. The ratio of leaf length to width and the degree of leaf dissection (number of leaflets per leaf length) both increased from the Western to the Eastern Alps (Fig. 11.5e,f). Mean SLA was highest in West Alpine plants. *Region* explained between 8.5–27.2% of variation in growth, 5.7–25.5% of variation in reproduction and 21.6–46.9% of the variation in leaf morphology (Table 11.4).

Populations within regions were significantly differentiated in six out of all 11 traits (Table 11.2). The covariate (initial plant diameter) had a significant influence on differentiation of several traits mainly related to growth (Table 11.2). *Block* had no significant influence on the investigated traits (results not shown). *Population* and *Genet* generally explained less variation than *Region*, with exception of reproductive traits (Table 11.4).

Table 11.3: Means (SE) for traits related to growth, reproduction and leaf morphology of genets derived 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 repr. meristems	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. 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^2mg^{-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 are not significantly different from one another at the $\alpha = 0.05$ level, using Tukey HSD post-hoc tests.

Table 11.2: Summary of linear mixed model analysis of genetic (region, population and genet) and environmental (competition) effects on growth, reproduction and leaf morphology of genets derived from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps

	Covariate		Competition		Region		Competition × Region		Population		Genet	
	MS	F_1	MS	F_1	MS	F_2	MS	F_2	s^2	$\chi^2{}_1$	s^2	$\chi^2{}_1$
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 repr. meristems	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	6696	9.9***	229	0.3	0.01	0.9	6.66	33.9***
Freq. 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: MS, Mean Squares and F, F-values are from conditional F-tests. Random effects: s^2, variances and χ^2, Chisquare values 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 the initial plant diameter, *Block* (random effect) was never significant and is not shown. Significance levels are represented by asterisks: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 11.4: Variance components V (%) of genetic effects (region, population and genet) on growth, reproduction and leaf morphology of genets derived 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 repr. meristems	7.7	4.0	20.9
Clonality	25.5	0.0	34.8
Freq. 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

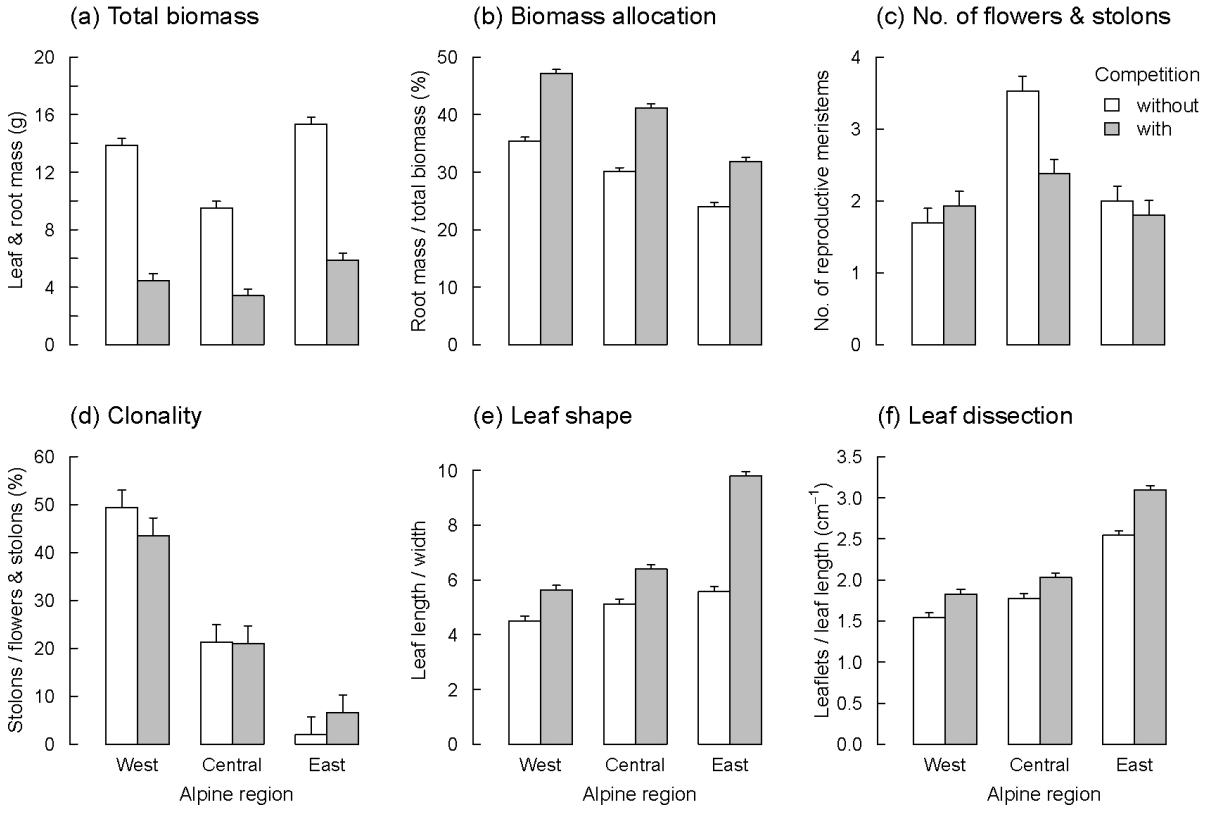

Figure 11.5: Quantitative trait differentiation in (a,b) growth, (c,d) reproduction and (e,f) leaf morphology of genets derived from 16 populations of *Geum reptans* from three different phylogeographic regions in the Alps. Genets were grown with and without competition from the grass *Poa alpina* in a common garden. Bars show means + SE based on the pooled error variance from ANOVAs. For significance of differences among regions see letters in Table 11.3.

Table 11.5: Estimates of quantitative trait differentiation (Q_{ST}) in growth, reproduction and leaf morphology as well as comparisons of Q_{ST} with neutral molecular differentiation (G_{ST}) of genets from 16 populations of *Geum reptans* sampled across 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 repr. meristems	0.141 (0.116–0.166)	<
Clonality	0.091 (0.079–0.104)	<
Freq. 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} was significantly different from G_{ST} (mean = 0.395, 95% CI 0.388–0.399), indicating diversifying (>) or unifying (<) selection. Neutral drift (=) is indicated when Q_{ST} was equal to G_{ST}.

Differentiation in growth response to competition

The competition treatment affected all plant traits significantly, with exception of clonality (Table 11.2). Only one significant interaction *Competition* × *Region* was found in the ratio of leaf length to width (Table 11.2), indicating that this trait responded differently to competition among regions. The effect of competition was found to be negative for most traits, but positive for allocation of biomass to the roots, for the ratio of leaf length to width and the degree of leaf dissection (Table 11.3; Fig. 11.5). Strong regional differentiation in competitive ability was observed concerning root mass (ANOVA (*Region*): $F_{2,15} = 3.52$, $P < 0.05$). Plants originating from the Central Alps had the highest relative competitive ability in root mass (0.73 ± 0.04, mean ± SE) compared with those of the other Alpine regions (Fig. 11.3b). Similar but non-significant trends in competitive ability were observed in leaf mass (results not shown).

Neutral drift versus adaptation

Q_{ST}-G_{ST} comparisons indicated that trait differentiation resulted not only from neutral genetic drift but from past selection as well (Table 11.5). Q_{ST} values were significantly different from G_{ST}, with 95% confidence intervals of Q_{ST} not overlapping those of G_{ST} in all traits with the exception of the ratio of leaf length to width. Significant correlations of traits with climatic data and altitude of population origin were found (Table 11.6), suggesting adaptation. Total biomass, leaf mass and root mass, and also the number of leaves correlated positively with annual maximum temperature, while all mentioned traits but root mass, correlated negatively with annual total precipitation (Fig. 11.6a). None of the assessed reproductive traits were related to any of the tested environmental variables. Concerning leaf morphological traits, SLA correlated positively with annual maximum temperature (Fig. 11.6b). Finally, allocation of biomass to the roots correlated positively with altitude of population origin (Fig. 11.6c). When the effect of region was removed statistically, the positive correlations of traits with temperature and altitude were not significant anymore, but the negative correlations for total biomass, leaf mass and number of leaves with annual total precipitation remained significant (see Appendix Table 11.8).

Table 11.6: Correlations of climatic data and altitude of population origin with traits related to growth, reproduction and leaf morphology of genets from 16 populations of *Geum reptans* sampled across 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 repr. meristems	-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. 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 coefficients r; Prec, annual total precipitation; T_{mean}, T_{min}, T_{max}, annual mean, minimum and maximum temperature; T_{summer}, annual summer temperature (mean June–August); Alt, altitude. Climatic data are obtained from the WorldClim database (Hijmans et al. 2005) and are averages of the years 1950–2000. Significance levels are represented by asterisks: $^*P < 0.05$, $^{**}P < 0.01$

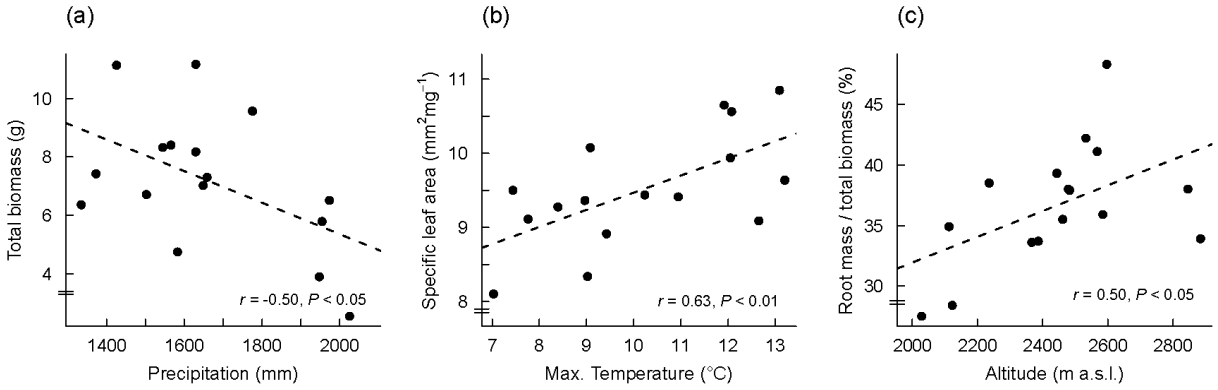

Figure 11.6: Correlations of phenotypic traits of *Geum reptans* with (a) annual total precipitation, with (b) maximum temperature and with (c) altitude of population origin. Genets from 16 populations of the species were sampled across the Alps and grown in a common garden. Climatic data are obtained from the WorldClim database (Hijmans et al. 2005) and are averages of the years 1950–2000. Shown in the graphs are correlation coefficients r and P-values from Pearson's correlation analyses.

Discussion

Molecular differentiation and phylogeograhic structure

We detected a relatively high genetic differentiation among populations of the perennial plant *G. reptans* ($G_{ST} = 0.40$) compared to the average value inferred from short-lived ($G_{ST} = 0.32$) or long lived ($G_{ST} = 0.19$) perennials from other RAPD studies reviewed in Nybom (2004). The high genetic differentiation in *G. reptans* is probably related to its particularly low seed dispersal ability in the Alpine landscape (Tackenberg and Stöcklin 2008). In addition, considerably high regional differentiation (36%) was derived from the AMOVA, which is higher than population differentiation (10%) and well in line with the distinct spatial genetic structure inferred from Bayesian cluster analysis.

The average genetic diversity ($H_e = 0.14$) within populations of *G. reptans* was rather low in comparison with genetic diversity in 20 other widespread species ($H_e = 0.22$; Nybom 2004).

Genetic bottlenecks due to small sizes of founder populations (Nei, Maruyama and Chakraborty 1975) during glacial survival or during postglacial remigration might explain the overall low level of genetic diversity. Moreover, genetic diversity was significantly lower in the West Alpine populations when compared with populations originating from the other two regions (Fig. 11.3a). The lower genetic diversity of West Alpine populations may be caused by the predominantly clonal reproduction of them (Fig. 11.5d), which has affected the genetic diversity negatively. On the other hand, the high genetic diversity of Central Alpine populations (Fig. 11.3a) may be due to the ongoing admixture of two previously separated gene pools.

The pronounced phylogeographic structure in *G. reptans* detected in our molecular data (Fig. 11.2) fits well with earlier findings of biogeographic studies in Alpine plants. The two main break lines splitting the Alps into three regions were already described based on floristic data (Merxmüller 1952; Ozenda 1988). A third break line partitioning the Central Alps into two regions, as suggested by researchers (e.g. Ozenda 1988), was not strongly supported by our findings of only one well separated Central Alpine group (Fig. 11.2b). We propose therefore that the Central Alpine group may have originated from an admixture of two originally separated gene pools in the middle part of the Alps. Two Central Alpine groups were found in another phylogeographic study with *G. reptans*, which included more populations in the cluster analysis and used AFLP markers (Thiel-Egenter et al. 2009). Our molecular data and the presence of several but only weakly supported molecular break lines in the Central Alpine region (see Fig. 11.4b in Thiel-Egenter et al. 2009) indicate that gene flow among the two Central Alpine groups is quite substantial probably due to a lack of pronounced dispersal barriers in the Central Alpine region. The significant isolation-by-distance pattern (Fig. 11.4) indicates that gene flow is more common among neighbours than distant populations within the entire Alpine belt and likewise within the Central Alps. The isolation-by-distance pattern supports the already mentioned low dispersal capacity of *G. reptans*.

The distinct west–east structure, as we observed in the silicicolous species *G. reptans*, concurs with proposed glacial refugia on silicious bedrock, longitudinally oriented at the 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 that occurred during its survival in glacial refugia. Subsequent weak gene flow among Alpine regions due to dispersal barriers, such as the deep valleys in the Western Alps (Aosta valley; Fig. 11.1) or the high mountain chains in the Eastern Alps (Grossglockner mountains; Fig. 11.1), most likely contributed to the regional differentiation in the widespread Alpine plant *G. reptans*.

Phenotypic differentiation

The present study is among the first that compares neutral molecular differentiation directly to phenotypic differentiation in the context of glacial history in the Alps. The regional structure derived from molecular markers fits well with the regional phenotypic differentiation in *G. reptans* observed in the common garden. In all assessed phenotypic traits and in growth response to competition, we detected strong regional differentiation (Tables 11.2 and 11.3; Fig. 11.3b). Moreover, the regional phenotypic differentiation was even higher than differentiation among populations within phylogeographic regions (Table 11.4).

The effects of initial plant diameter on phenotypic traits (Table 11.2) indicate that size differences at the beginning of the experiments influenced several traits significantly. Such initial differences in plant size could partly be due to maternal effects (Weiner et al. 1997). By using initial plant diameter as a covariate, these size differences should, therefore, not have affected the outcome of the tested genetic (region, population and genet) and environmental factors (competition).

Several main patterns of regional phenotypic differentiation were also found which are worth mentioning: i) the significantly lower vegetative biomass and enhanced reproductive output of Central Alpine plants in comparison to plants originating from the Western and the Eastern Alps (Fig. 11.5a,c); ii) the decrease in allocation of biomass to the roots and in clonality from

west to east, as well as the increase in the ratio of leaf length to width and in the degree of leaf dissection (Fig. 11.5b,d,e,f). Regional differentiation in phenotypic plasticity is also indicated, since Central Alpine plants suffered less from competition and had a higher competitive ability compared to the plants from the other two regions (Fig. 11.3b).
We suggest that a home advantage effect could explain the enhanced reproductive output and similarly 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 their original locations which may have enabled them to reach higher fitness compared to plants originating from other regions. However, a home advantage effect can hardly explain the reduced vegetative biomass of Central Alpine plants (which did not correlate negatively with the reproductive output) and the decrease of trait values from east to west or vice versa. Therefore, we conclude that other evolutionary processes in the past, either neutral or adaptive processes, may have played a role in regional differentiation in biomass, reproduction and leaf morphology of *G. reptans*.

Neutral drift and glacial history

Our results suggest strong historical effects including neutral genetic drift and subsequent limited gene flow during glacial survival and postglacial remigration which must have affected phenotypic differentiation in *G. reptans*. Since differentiation in the ratio of leaf length to width and in the number of leaflets per leaf length was especially strong in East Alpine plants, compared to those originating from the other regions (Table 11.3), either a separate more eastern gene pool or selection might have caused the strong differentiation in leaf morphology found in the Eastern Alps. It is important to note, when considering studies based on widespread plant species, that neutral evolutionary processes leading to differentiation in quantitative traits are frequently neglected (e.g. Joshi et al. 2001; Olsson and Ågren 2002). We emphasise here the relevance of such neutral processes for phenotypic differentiation, and we suggest, as a consequence of glaciations in the Alps, that neutral phenotypic differentitation might be a more general phenomenon in widespread Alpine plants than previously assumed.

Indication for adaptation

Besides neutral evolution, part of the observed phenotypic differentiation in *G. reptans* may be explained by historical selection from environmental conditions during survival in glacial refugia outside the Alps or by selection from current environmental conditions during recolonisation of the Alps. Past selection ($Q_{ST} \neq G_{ST}$) is indicated in almost all traits (Table 11.5). Thus selection played an important role in shaping the observed phenotypic differentiation, suggesting a relatively high adaptive potential in growth, reproduction and leaf morphology of the Alpine plant species *G. reptans*. Adaptation causing strong phenotypic differentiation is also known from widespread lowland species (Joshi et al. 2001; Becker et al. 2006).
In order to prove any adaptation to particular local conditions, reciprocal transplantation experiments would be needed (Kawecki and Ebert 2004). However, in our study adaptation is suggested based on the significant correlations of several traits measured in the common garden with the climatic data and altitude of population origin (Table 11.6). For example, biomass and SLA correlated with climatic variables at the original sites of populations. The reduction in biomass with decreasing temperature might thus be an adaptive strategy of plants of *G. reptans* from locations with low temperatures to reduce freezing damage, as observed in other Alpine plant species (Körner 2003). In addition, the reduced biomass in plants from locations with high annual total precipitation (Fig. 11.6a), which also includes snowfall, could be an adaptation of plants in growth to an extended snow cover and a shortened growing season. The negative correlation of biomass with precipitation remained even when the regional effect was removed statistically (see Table 11.S2), emphasising the importance of precipitation in local adaptation of traits related to plant growth. Reduced SLA values (i.e. greater leaf thickness) in plants originating from locations with low temperatures (Fig. 11.6b) are probably an adaptation to climatic variation as well (e.g. Scheepens, Frei and Stöcklin 2010). Adaptation to environmental conditions related to altitude is

indicated by a significant positive correlation between altitude of population origin and allocation of biomass to the roots (Fig. 11.6c). This positive correlation could be due to an increase in fine-root mass with altitude as a substitution for the reduced mycorrhizal infection at high altitudes in *G. reptans* (Nespiak 1953; Körner and Renhardt 1987). Although Q_{ST}-G_{ST} comparisons indicated selection for reproduction, none of the reproductive traits correlated with the climatic data or altitude of population origin. Therefore, differentiation in reproduction could partially be explained by adapation to historical conditions or to current environmental conditions, which were not measured in our study.

Conclusions

In the present study, we used a phylogeographic approach to investigate regional phenotypic differentiation in the widespread Alpine plant *G. reptans* at a large spatial scale (European Alps). We demonstrated that the phylogeographic structure inferred from neutral molecular markers is mirrored by similar and strong regional phenotypic differentiation in this species. Our results suggest that historical forces, such as neutral drift and subsequent limited gene flow during survival in glacial refugia and postglacial remigration, affected differentiation in both genotypes as well as phenotypes. From a Q_{ST}-F_{ST} comparison and correlations between biomass and precipiation data of population origin, we conclude that adaptive processes to climatic differences may explain, at least partially, the observed phenotypic differentiation in the widespread *G. reptans*. Based on our results, we suggest that drastic historical range changes during climatic oscillations and Alpine glaciations in the Quaternary have left their mark in phenotypic differentiation patterns of common plant species. Therefore, the present study might be of relevance for estimating the adaptive potential and the consequences of future range changes of Alpine plants in response to global climate change.

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Appendix

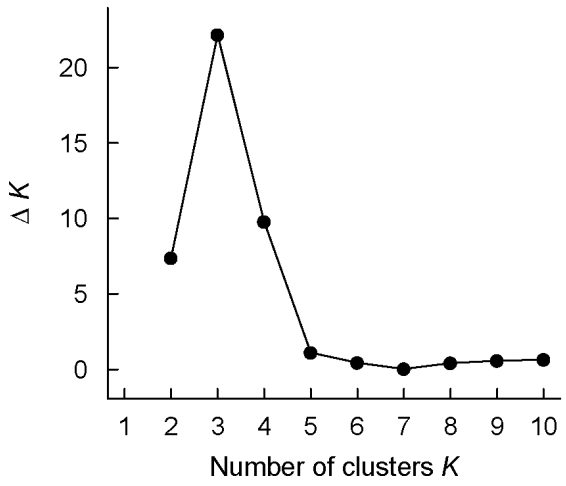

Figure 11.7: Ad-hoc statistics ΔK from STRUCTURE analysis.

Table 11.7: Sites of populations of *Geum reptans* sampled across the European Alps with climatic data and altitude

Alpine region	Location	Pop.	°North / °East (WGS84)	Prec (mm)	T_{mean} (°C)	T_{min} (°C)	T_{max} (°C)	Alt. (m a.s.l.)
West	Valfroide, near Lac du Goléon	GOL	45.08200/6.36250	1544	0.4	-10.2	13.1	2443
West	South of Col du Galibier	GALS	45.06278/6.40444	1629	-0.3	-10.4	12.1	2567
West	North of Col du Galibier	GALN	45.06303/6.40833	1629	-0.4	-10.6	11.9	2596
West	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
Central	Foreland of Mutt glacier	MUT	46.55750/8.41250	195	-1.7	-11.4	9.0	2482
Central	Furkapass, Blauberg	BLA	46.56917/8.41750	2025	-1.9	-11.6	8.4	2532
Central	Foreland of Steinlimi glacier	STE	46.71167/8.41833	1973	0.3	-9.5	10.9	2112
Central	Val Fex	FEX	46.36111/9.79528	1582	0.4	-8.6	10.2	2235
Central	Foreland of Scaletta glacier	SCA	46.70361/9.93722	1565	-0.5	-9.8	9.4	2366
Central	Flüelapass	FLU	46.74583/9.94556	1502	-1.2	-10.5	9.1	2478
Central	Vadret da Cambrena	CAM	46.40417/9.99917	1648	-0.5	-9.3	9.0	2386
Central	Foreland of Tuoi glacier	TUO	46.84000/10.14333	1658	-1.9	-11.0	7.8	2584
Central	Foreland of Pitztal glacier	PIT	46.92750/10.87806	1372	-3.6	-13.3	7.0	2884
Central	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
East	Schladminger Tauern	STAU	47.27508/13.75867	1526	-0.1	-11.4	12.7	2070
East	Triebener Tauern	TTAU	47.39409/14.53531	1441	-0.9	-12.8	12.8	2126

Populations are grouped by phylogeographic regions as inferred from a Bayesian cluster analysis with molecular data. Populations are ordered from Western (top) to Eastern Alps (bottom). Annual precipitation data (Prec) and temperature data (T_{mean}, T_{max}, T_{min}) are obtained from the WorldClim database (Hijmans et al. 2005) and are averages of the years 1950–2000.

Table 11.8: Correlations of climatic data and altitude of population origin with phenotypic traits of genets from 16 populations of *Geum reptans* sampled in 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 repr. meristems	-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. 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	0.1	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 coefficients r. Prec, annual total precipitation; T_{mean}, T_{min}, T_{max}, annual mean, minimum and maximum temperature; T_{summer}, annual summer temperature (mean June–August); Alt, altitude. Climatic data are obtained from the WorldClim database (Hijmans et al. 2005) and are averages of the years 1950–2000. Significance levels are represented by asterisks: $^*P < 0.05$.

Chapter 12

Latitudinal and altitudinal differentiation in phenotypic traits and molecular markers of *Campanula rotundifolia*

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In preparation for *Conservation Genetics*

Latitudinal and altitudinal differentiation in phenotypic traits and molecular markers of *Campanula rotundifolia*

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Abstract

Plant species will resist to climate change only if they are able to migrate or to adapt to a warmer climate in sufficient time. However, the potential for plant species to adapt is mostly unknown. This adaptation potential is indicated by substantial heritability in phenotypic traits. Q_{ST}–G'_{ST} comparisons, which can reveal past selection pressures on phenotypic traits, and correlations of trait values with climatic conditions, which suggest current adaptation to climate, can be used to indicate the necessity for phenotypic traits to be adapted to environmental conditions. In this study we performed a common garden experiment, correlations with climatic conditions and Q_{ST}–G'_{ST} comparisons to assess the need and potential for future adaptation in phenology, morphology and fitness traits in European populations of *Campanula rotundifolia* L. (Campanulaceae). We grew plants from nearby populations (Central Europe), as well as plants from populations from The Netherlands, Scandinavia and from the European Alps in the common garden in Basel, Switzerland, and applied a competition treatment. All twelve investigated phenotypic traits showed past selection pressure, a significant regional differentiation and substantial heritability (0.496 ± 0.068). Start of flowering and flowering duration were likely adapted to season length. The home site populations showed the fittest performance in above-ground biomass and reproductive output, probably due to common garden effects. The effect of competition treatment varied stronger within regions than among regions, which can be explained by the heterogeneous distribution in competition pressure. This study showed that investigated traits of *Campanula thyrsoides* are likely adapted to environmental conditions, with phenological traits being adapted to climate, and that all traits have the potential to adapt to climate change.

Keywords: common garden, latitudinal and altitudinal variation, RAPDs, Q_{ST}-G'_{ST} comparison

Introduction

The currently rapid climate and land-use changes (IPCC et al. 2007; Vitousek et al. 1997) pose a threat to the survival of plant populations. At present, loss of genetic variability is above the natural background extinction rate (Vitousek et al. 1997). To escape that threat of extinction plants have to adapt to climate change, whether through migration, genetic adaptation or phenotypic plasticity (Brad-

shaw 1984; Joshi et al. 2001). In Europe that generally means adaptation to increased mean temperatures and to an increased frequency of summer droughts (IPCC et al. 2007). Many species respond by shifting their range poleward and to higher elevations in order to track climate belts, though this also may require a considerable adaptation potential (Davis and Shaw 2001; Walther 2004). Moreover, it is proposed that for many species the necessary migration speed to track climate change is too fast for plant populations, mainly due to their limited seed dispersal (Davis and Shaw 2001; Jump and Peñuelas 2005). Additionally, range shifts lead to an alteration of biotic interactions (van der Putten et al. 2004; Engelkes et al. 2008), thus plants probably would have to compete in an environment with new competitors.

The possibility for phenotypic traits to adapt to the environment implies that traits should be at least partly genetically based and heritable (Houle 1992). In our study, adaptation was explored by investigating genetic differentiation in phenotypic traits among regions and populations and by examining whether any differentiation correlated with the climate of origin (Linhart and Grant 1996). The future potential of phenotypic traits to adapt to changing environments was investigated by assessing the heritability. Common garden experiments were used to reveal genetic differentiation and heritability (Turesson 1923; Clausen et al. 1941; Galen et al. 1991; Olsson and Ågren 2002). Our sampling design with Central European home site populations, i.e. originating from the same region as the experimental garden, as well as foreign populations from higher latitudes and altitudes resembled a reciprocal transplantation design. With such a design local adaptation can be concluded if home populations perform better than foreign populations (Rice and Mack 1991).

Correlations of trait values with climatic conditions at the sites of origin suggest adaptation, but it is not a definite proof. Although differentiation observed in the common garden experiment may correlate with environmental factors, this may also be the result of genetic drift. Whether traits were subject to selection pressures in the past can be established when quantitative differentiation (Q_{ST}) deviates from neutral molecular differentiation (G'_{ST}). If a trait differentiated neutrally, theory predicts that $Q_{ST} = G'_{ST}$, while $Q_{ST} > G'_{ST}$ is predicted under diversifying selection, and $Q_{ST} < G'_{ST}$ is predicted under unifying selection (Spitze 1993; Merilä and Crnokrak 2001; McKay and Latta 2002).

The phenology of widespread species often shows variation along climatic clines, for instance along latitudinal and altitudinal gradients, suggesting adaptation to continuously changing climatic conditions (Briggs and Walters 1997; Clevering et al. 2001; Olsson and Ågren 2002). Common garden experiments showed that with increasing latitude initiation of flowering advances and the growing and flowering period shortened for *Lythrum salicaria* (Olsson and Ågren 2002; Montague et al. 2007). A similar pattern of earlier initiation of flowering and decreasing length of growth and flowering period was also found with increasing altitude for *Potentilla glandulosa* (Clausen et al. 1947). However, for *Campanula* species Blionis and coworkers (2001) revealed a delayed initiation of flowering and no decrease in flowering duration with altitude on Mt Olympos in Greece.

In contrast to adaptation to gradients, the ability to withstand competition should rather show local adaptation according to the mosaic-like pattern of competition within the landscape, i.e. not necessarily according to the sampled climatic cline. Such a heterogeneous patterns of local adaptation to fine-grained environmental conditions has, for instance, been shown for soil type, local stress factors or pollinators (Antonovics 1971; MacNair et al. 1993; Linhart and Grant 1996; Thompson and Cunningham 2002).

In this study, the goal was to investigate the potential of the harebell, *Campanula rotundifolia* L., to adapt to future climatic changes. This perennial herb is widespread in the temperate zone of the northern hemisphere. It has been shown that *C. rotundifolia* is sensitive to land use changes (Lindborg et al. 2005) and that its distribution is rapidly declining in The Netherlands ("Floron Nieuws" 2007). A common garden experiment with *C. rotundifolia* populations from regions at different latitude and altitude was performed to reveal regional differentiation in various phenotypic traits and to measure their

heritability. We estimated competition ability by adding a competitor to the common garden experiment as a treatment. Additionally, an analysis of the neutral genetic structure, using RAPDs as molecular markers, was performed to give insight into the spatial genetic structure (AMOVA) and to reveal past selection pressures on the investigated traits (Q_{ST}–G'_{ST} comparisons). We hypothesised that (1) genetic differentiation occurs across the latitudinal as well as the altitudinal regions for traits which are associated with climate, such as start of flowering, flowering duration, and that (2) genetic differentiation is high among populations within regions, rather than among regions, for phenotypic traits expected to be adapted to more locally heterogeneous environmental conditions, such as competition ability. We further aimed to assess the potential to adapt to future climate change by investigating heritability of phenotypic traits, and to examine whether the traits have been subject to selection in the past by performing Q_{ST}–G'_{ST} comparisons.

Methods

Study species and sampling design

The perennial herb *Campanula rotundifolia* L. (Campanulaceae) has a broad distribution in the Northern hemisphere. In Europe the species occurs from Spitsbergen (Engelskjon et al. 2003) to Greece (Blionis et al. 2001) and from sea level to subalpine zones; predominantly in semi-natural grasslands, but also in ruderal sites (Podlech 1962; Lauber and Wagner 1998). Within a growing season the plant forms a rosette which partly dies back during flowering. One to several flowering stems appear with two or more violet, bell-shaped flowers per stem. The self-incompatible plant is insect-pollinated, usually by bees (Shetler 1982; Bingham and Orthner 1998), its seeds are dispersed by wind and water (Shetler 1982; Nyman 1992) and clonal reproduction has also been observed occasionally (Lindborg et al. 2005).

For the common garden experiment, seeds were sampled from 19 populations in four regions (Fig. 12.1, Table 12.1). A total of 111 individuals were sampled, whose progenies were referred to as seed families. Seed capsules and leaf material were sampled within each population with a minimum distance of one meter between each sampled plant. Leaf material was dried and stored with silica gel until DNA extraction. From North to South regions included Scandinavia (5 populations), The Netherlands (5 populations) and Central Europe (5 home site populations). The fourth region was the Swiss Alps at high elevation (4 populations). During the growing season the Alpine regions are characterized by generally cool and wet conditions, while in Scandinavia a cool and dry weather prevails in summer compared with the Central European conditions (Table 12.1). In order to get a balanced design for the molecular marker analysis only four out of five populations per region were considered (see Table 12.1).

Figure 12.1: Map with sampling locations. Five *Campanula rotundifolia* populations in SC (Scandinavia, Denmark and Sweden), five populations in NL (The Netherlands), five populations in CE (Central Europe, Switzerland and France) and four populations in AL (Swiss Alps); detailed description of the population sites are given in Table 12.1.

Table 12.1: Sampled *Campanula rotundifolia* populations used for a common garden experiment and molecular analysis with geographic coordinates and the regional mean temperature and precipitation over the months June, July and August.

Region	Pop	Country and population name	°North	°East	Alt	T_{mean}	Prec
Scandinavia	SC_1	SE, Höganäs, Kullen	56.29	12.47	30	16 - 16.3	170 - 204
"	SC_2	DK, Kalundborg, Gniben	55.92	11.08	5		
"	SC_3	DK, Kalundborg, Vesterlyng	55.73	11.25	5		
"	SC_4	DK, Stevns, Odden	55.43	12.22	5		
"	SC_5[a]	SE, Höganäs, Haga	56.28	12.56	20		
The Netherlands	NL_1	NL, Drehnte, Anloo	53.05	6.69	10	15.6 - 16.3	217 - 228
"	NL_2	NL, Drehnte, Gasterse Duinen	53.04	6.66	10		
"	NL_3	NL, Utrecht, Utrecht	52.10	5.12	5		
"	NL_4	NL, Gelderland, Rozendaalse bos	52.03	5.95	60		
"	NL_5[a]	NL, Gelderland, Kievitsdel	51.98	5.77	30		
Central Europe	CE_1	CH, Basel-Landschaft, Sulzkopf	47.50	7.66	480	15.3 - 17.5	296 - 330
"	CE_2	CH, Solothurn, Hofstetter Chöpfli	47.49	7.50	520		
"	CE_3	CH, Basel-Landschaft, Blauen	47.49	7.53	620		
"	CE_4	FR, Alsace, Kiffis	47.45	7.38	620		
"	CE_5[a]	CH, Solothurn, Hochwald	47.47	7.52	500		
Alps	AL_1	CH, Graubünden, Haldenstein	46.88	9.53	620	9.1 - 17.5	378 - 458
"	AL_2	CH, Graubünden, Davos	46.78	9.81	1580		
"	AL_3	CH, Uri, Göschenen	46.67	8.59	1100		
"	AL_4	CH, Bern, Gündlischwand	46.64	7.92	1000		

[a] Population not used in the molecular analysis.

Common garden experiment

The common garden experiment was performed in the Botanical Garden of Basel, Switzerland (N 47°33'29.98", E 7°34'52.05", 270m a.s.l.). In March 2009 the seeds from previously collected capsules were germinated on water-saturated filter paper in petri dishes. The petri dishes were placed for 20 days in a growing chamber (12 h light / 12 h dark; 20°C / 10°C), moistened daily if necessary and randomised twice a week. When the seedlings reached the one-leaf stage, they were transplanted in pairs in trays with 54 pots (5 cm Ø x 5 cm height filled with low nutrient soil). The plants were protected against drought with a fine mesh cloth directly above the pots. The seedlings were watered daily and trays were randomized twice a week. Early in May, one of two seedlings per pot was transplanted into a larger pot (10 × 10 × 10 cm). After adjusting the number of individuals per seed family to balance the design at the population level, each population contained 56 individuals out of two to seven seed families depending on the available number of seed families, resulting in 1064 individuals from 19 populations (Table 12.1). Half of the individuals per seed family received a competition treatment, viz. four seedlings of *Bromus erectus* Huds. s.str. (Poaceae) were added in the corners of the pots. *B. erectus* is a grass species widespread in Europe and frequently co-occurring in vegetation communities with *C. rotundifolia*. Its vigorous growth makes it a suitable competitor for this experiment.
One week after the transplantation in the greenhouse, on 14th May 2009, all pots were placed outdoors in the Botanical Garden of Basel, in trays holding 24 pots. The plants were watered regularly, protected from snails using Ferramol® grains (Biocontrol, Andermatt, Switzerland), and trays were randomised twice a week. Two to three times a week the date of the first opened flower per plant was recorded as well as the date of the end of flowering per plant, i.e. when all flowers had withered. From each plant the length and width (diameter) of the corolla from a medium sized flower was measured. From 1st – 9th July 2009 rosette leaves were counted, and the length, width and thickness of a mature leaf as well as the flowering stem length were measured. As part of the vegetative biomass analysis, the shedded rosette leaves were regularly sampled in order to include litter in final biomass analysis. To assess reproductive output, ripe capsules were collected and counted throughout the season. Due to the porosity of ripe capsules, they might have lost seeds before

sampling. Therefore the number of seeds was not a reliable measure of reproductive output. As soon as the flowering season finished and the plants started to wither, the number of flowering stems was counted and above-ground biomass of the plants was harvested and dried for 72 h at 60° C. The above-ground vegetative biomass was weighed including litter and excluding capsules and flowers.

Data analysis

For statistical analyses of continuous traits hierarchical generalised linear mixed models (GLMM) were performed with the software package R (Version 2.10.1, R.Development Core Team 2009; package *lme4*, Bates and Maechler 2009). The following fixed factors were tested: competition, region, and competition × region interaction. The random factors were: tray, population nested in region, seed family nested in population and the interactions of competition with population and with seed family. Survival and flowering were analysed using a binomial distribution. All other response variables fitted better to a normal distribution. To improve the normality of the model residuals, we used natural logarithmic transformation for above-ground biomass, number of capsules, number of leaves and number of stems and a square root transformation for length of stems. Pearson's correlations between phenotypic traits and latitude, altitude, temperature means and precipitation were tested for significance, which would suggest that traits are adapted to these environmental factors. Climate data was obtained from WorldClim (Hijmans et al. 2005) and mean temperature and precipitation were calculated for the summer months (June, July and August) from 1950 to 2000 (Table 12.1). Of the four WorldClim data points surrounding a sampling site, the point with the least altitudinal difference to the location of origin was chosen for each population, and the temperature values were corrected for altitude by subtracting or adding 0.0055°C per meter difference (Ozenda 1988).

Competition ability

The response of plants to competition was calculated as unity minus the proportional reduction in plant size of individuals from one seed family treated with *Bromus erectus* compared to individuals from a seed family growing without competitor (after Snaydon 1991). Thus, the lower this measure of relative competition ability, the stronger is the negative effect of competition on seed family performance. To investigate the variability in competition ability among populations and regions, the coefficient of variation was calculated among and within regions. Additionally, the correlation between competition ability and genetic diversity was tested for significance.

Molecular diversity

Genomic DNA was extracted from dried leaf material of 13 individuals from each of the 16 selected populations (Table 12.1) following Pluess and Stöcklin (2004). DNA concentration was measured using NanoDrop® (ND-1000, Witec, Littau) and adjusted to 1 ng μL^{-1}. After a pilot analysis with ten different RAPD primers, the following five primers were chosen for fingerprinting based on their high polymorphism, quantity and quality of bands: X5 = CggTCACTgT, M6 = gTgggCTgAC, H2 = TCggACgTgA, H13 = gACgCCACAC, and H20 = gggAgACATC (Eurofins MWG Synthesis, Ebersberg, Germany). RAPD-PCR was performed with self-dissolving illustraTM 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 ca. 2.5 U polymerase. The beads were dissolved in 18.75 μL ddH20, and 0.25 μL of each primer and 6 μL of genomic DNA were added before the amplification. PCRs were run in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with the following cycling scheme: 2min at 94°C for initial denaturation, followed by 34 cycles of 30sec at 92°C, 30sec at 36°C and 1.5min at 72°C, and a final extension step of 5min at 72°C. The PCR products were separated on 2% agarose gels in 0.5×Tris-Borate-EDTA buffer with several 100bp DNA ladders

(Fermentas, St. Leon-Rot, Germany) as size standard. Clear and distinct bands were scored after staining the gels with ethidium bromide. Size range of PCR fragments was between 250bp and 1900bp. All analysed individuals yielded readable RAPD profiles. Repeatability of the banding patterns, tested on eleven duplicated individuals, was 95%, with 16 out of 374 scored bands classified as false positive or false negative.
RAPD patterns were coded as a zero/one matrix in GenAlEx, version 6.2 (Peakall 2006). Both polymorphic and monomorphic bands were taken into account (Nei 1973). The GenAlEx file was then used for several population genetic tools. Popgene version 1.32 (Yeh and Boyle 1997) estimated Nei's expected genetic diversity for each population as well as pairwise genetic distances between all pairs of populations. Isolation by distance was calculated using a Mantel test (Mantel 1967). GenAlEx was also used to estimate the molecular variance (amova) among regions and among populations within regions. The program Structure v2.2 (Falush et al. 2003) and the program tfpga v1.3 (Miller 1997) were used to investigate the spatial genetic structure. Structure was run with an admixture model without prioir population information and witg putatively independent allele frequencies in each population. Burn-in and MCMC was set to 10'000 and 100'000 iterations, respectively. Log-likelihood of K=1-7 clusters were computed each with 10 replicates.

Heritability and past selection pressure

Narrow-sense heritability h^2 was calculated following Petit and co-workers (2001) based on the half-sib design using the following formula:

$$h^2 = 4\,V_{\mathrm{FAM}} \,/\, (4\,V_{\mathrm{FAM}} + V_{\mathrm{POP}})$$

where V_{POP} corresponds to the variance among populations and V_{FAM} the variance among seed families within populations. Quantitative differentiation among populations, Q_{ST}, was calculated for each trait using the following formula under the assumption that the progenies of a maternal plant were half-sibs (Petit et al. 2001):

$$Q_{\mathrm{ST}} = V_{\mathrm{POP}} \,/\, (8\,V_{\mathrm{FAM}} + V_{\mathrm{POP}})$$

Neutral marker differentiation can be compared with quantitative trait differentiation to infer past selection pressures (Wright 1951; Spitze 1993). Among-population marker differentiation, G_{ST}, was calculated with Popgene (Yeh and Boyle 1997). The modified G'_{ST} index was then calculated manually using G_{ST}, H_{S} (heterozygosity within populations) and the number of populations (Hedrick 2005, equation 4b). The standard deviations of Q_{ST} and G'_{ST} were estimated using jackknifing among populations, and differences were tested with t-tests.

Results

Phenotypic Differentiation

During the common garden experiment 28% of the plants died, and survival showed a significant decrease with higher latitude and altitude of origin (Tables 12.2 and 12.3). A total of 65% of the surviving individuals flowered and the percentage of flowering individuals decreased likewise significantly towards higher latitude and altitude (Tables 12.2 and 12.3).
The flowering period of experimental plants lasted from 4$^{\mathrm{th}}$ June to 20$^{\mathrm{th}}$ October (average = 35 ± 4 d). The mean flowering duration of Scandinavian plants was significantly shorter than those from The Netherlands and Central Europe (Fig. 12.2a). The start of flowering ranged from 4$^{\mathrm{th}}$ June to 20$^{\mathrm{th}}$ September with a peak of individuals starting to flower at 26$^{\mathrm{th}}$ July. The Scandinavian plants showed a delayed initial flowering compared to lower latitudes, and Alpine plants showed the earliest flowering (Fig. 12.2b). The fitness traits of above-ground biomass and number of capsules were approximately twice as big in the Dutch and Central European plants as in Scandinavian and Alpine plants (Fig. 12.2c and d).
Morphological traits also showed regional differentiation (Tables 12.2 and 12.3). The number of rosette leaves was significantly higher for the Dutch plants compared to the Central European plants, and the number and length of flowering stems decreased with latitude and altitude. The leaf thickness also revealed a decrease towards colder regions. The leaf shape (length/width) was elongated in Scandinavian

and Alpine plants, whereas Central European and Dutch plants had wider leaves.
Although all observed traits showed trends with increasing latitude and altitude, only some traits showed a significant correlation with latitude or altitude of origin, viz. the fitness traits above-ground biomass and number of capsules, the initial flowering and some of the morphological traits (Appendix Table 12.8). The traits that correlated with latitude and altitude also showed significant correlations with the climate variables at the sites of origin: temperature mean and precipitation sum during summer months. The flowering duration and flower shape showed non-significant patterns.

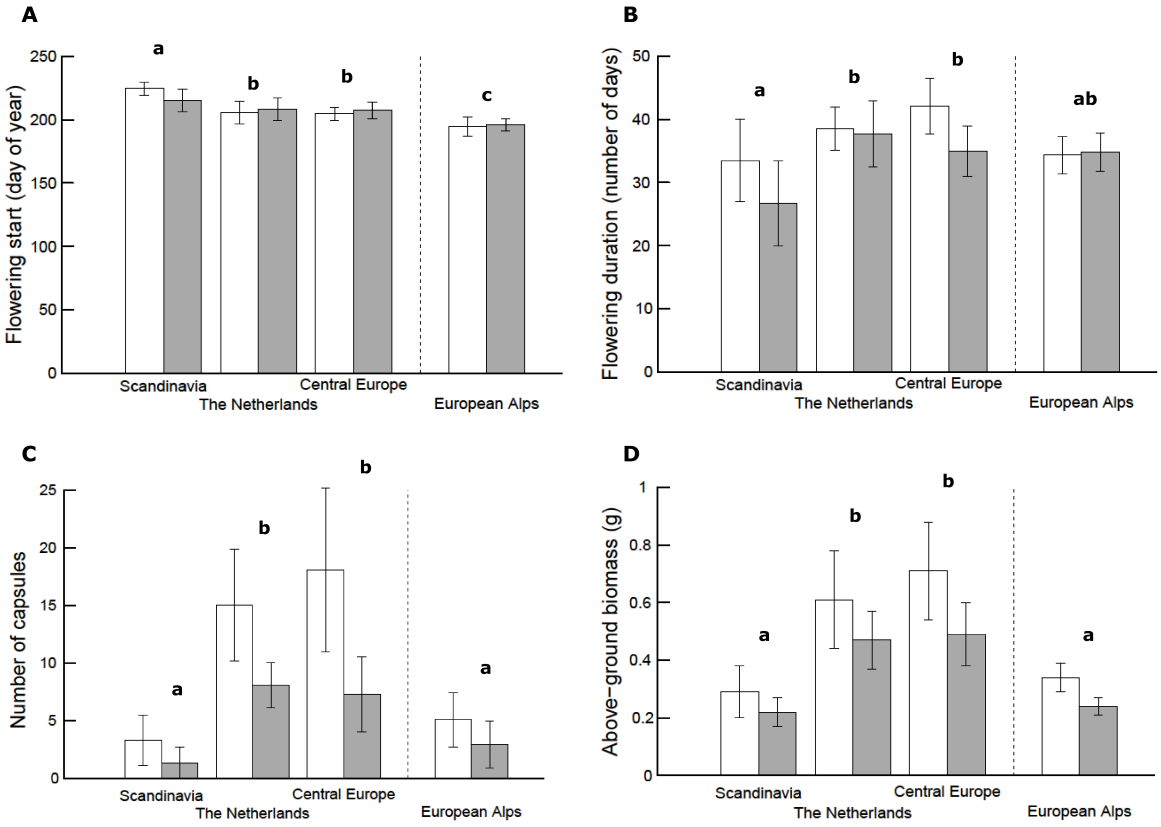

Figure 12.2: Map with sampling locations. Five *Campanula rotundifolia* populations in SC (Scandinavia, Denmark and Sweden), five populations in NL (The Netherlands), five populations in CE (Central Europe, Switzerland and France) and four populations in AL (Swiss Alps); detailed description of the population sites are given in Table 12.1.

Response to competition

The competition treatment had a significant effect on 6 out of 12 traits (Table 12.2). Interestingly, the plants growing with competitors survived better in the common garden experiment, but as expected competition showed a negative effect in above-ground biomass, number of capsules, flowering duration, number of stems and stem length (Table 12.3). The competition × region interaction showed significant differences in competition ability among regions in the number of capsules and in leaf thickness (Table 12.2). The number of capsules decreased stronger in Central European and Dutch plants when grown with competition, compared to the other regions (Fig. 12.2d). In Scandinavian and Dutch populations leaf thickness was not

Table 12.2: Results of GLMM analysis of the effects of tray (random), competition (C; fixed), region (R; fixed), populaton (P; random), seed family (Sd; random) and of the interaction effects: competition × region (C×R; fixed), competition × population (C×P; random) and competition × seed family (C×Sd; random) on 12 phenotyic traits of *Campanula rotundifolia* in a common garden. Chi^2 with significancies were obtained from model camparisons.

	df	Survival		Flowering		Biomass		Number of capsules		Flowering start		Flowering duration		Number of leaves		Number of stems		Stem length		Leaf thickness		Leaf shape		Flower shape	
Tray	1	82	***	53	***	98	***	41	***	48	***	14	***	114	***	179	***	112	***	102	***	3.8	*	2.1	
C	1	11	***	3.3		62	***	46	***	2.8		13	***	0.2		63	***	21	***	3.3		0.2		1.6	
R	3	167	***	172	***	271	***	62	***	75	***	13	***	20	***	213	***	219	***	13	***	26	***	8.5	*
P	1	43	***	17	***	96	***	27	***	60	***	1.3		18	***	37	***	93	***	8.8	**	3.3		0	
Sd	1	52	***	7.0	**	21	***	5.1	*	12	***	8.5	***	49	***	23	***	32	***	9.5	**	3.7		0	
C × R	3	3.3		2.4		7.1		11	*	4.4		7.8		4.0		4.1		4.5		11	*	1.6		1.9	
C × P	1	0		0.3		4.1	*	0.1		0.5		0		0.2		0.7		0.9		0.8		2.3		0	
C × Sd	1	0		0		1.2		2.1		8.7	**	6.7	**	4.4	*	0.5		2.2		1.4		0.9		0	

* $P = 0.05 - 0.01$, ** $P = 0.01 - 0.001$, *** $P < 0.001$

Table 12.3: Regional and competitive mean values for 12 phenotypic traits of *Campanula rotundifolia* per region (SC: Scandinavia, NL: The Netherlands, CE: Central Europe, AL: Swiss Alps) and competition treatment (- plants grew solitary, + plants grew with *Bromus erectus* as competitor) in a common garden.

		Survival (%)	Flowering (%)	Biomass (g)	Number of capsules	Flowering start (day of year)	Flowering duration (days)
Region	SC	56.9 ± 19.6 ^{a}	21.9 ± 12.9 ^{a}	0.26 ± 0.07 ^{a}	2.3 ± 1.66 ^{a}	220 ± 7 ^{a}	30.1 ± 5.1 ^{a}
	NL	71.7 ± 14.8 ^{b}	63.8 ± 20.7 ^{b}	0.54 ± 0.14 ^{b}	11.55 ± 3.50 ^{b}	207 ± 10 ^{b}	38.1 ± 4.4 ^{b}
	CE	95.4 ± 4.8 ^{c}	84.9 ± 14.0 ^{b}	0.60 ± 0.15 ^{c}	12.67 ± 5.63 ^{b}	206 ± 6 ^{b}	38.5 ± 3.5 ^{b}
	AL	70.0 ± 11.8 ^{b}	60.1 ± 12.7 ^{b}	0.29 ± 0.04 ^{a}	4.01 ± 2.34 ^{a}	196 ± 7 ^{c}	34.6 ± 2.6 ^{ab}
Competition	–	68.7 ± 19.3 ^{a}	59.3 ± 27.4 ^{a}	0.49 ± 0.20 ^{a}	10.37 ± 7.29 ^{a}	208 ± 13	37.1 ± 4.0 ^{a}
	+	78.3 ± 12.8 ^{b}	56.0 ± 25.3 ^{b}	0.36 ± 0.15 ^{b}	4.89 ± 3.28 ^{b}	207 ± 8	33.5 ± 4.7 ^{b}

		Number of leaves	Number of stems	Stem length (mm)	Leaf thickness (mm)	Leaf shape (leaf length/width)	Flower shape (flower length/width)
Region	SC	10.8 ± 2.3 ^{a}	1.5 ± 0.6 ^{a}	34 ± 12 ^{a}	0.18 ± 0.03 ^{a}	0.98 ± 0.11 ^{a}	1.08 ± 0.04
	NL	11.1 ± 0.8 ^{b}	3.3 ± 0.8 ^{b}	152 ± 74 ^{b}	0.18 ± 0.03 ^{a}	0.89 ± 0.06 ^{ab}	1.04 ± 0.09
	CE	8.3 ± 1.0 ^{b}	3.2 ± 1.0 ^{b}	163 ± 54 ^{c}	0.21 ± 0.01 ^{b}	0.87 ± 0.03 ^{b}	0.98 ± 0.07
	AL	10.3 ± 1.1 ^{b}	2.7 ± 0.4 ^{a}	128 ± 31 ^{d}	0.19 ± 0.04 ^{a}	0.88 ± 0.10 ^{b}	1.04 ± 0.08
Competition	–	10.2 ± 1.4	3.0 ± 0.9 ^{a}	131 ± 65 ^{a}	0.19 ± 0.02	0.90 ± 0.04	1.02 ± 0.02
	+	10.1 ± 1.3	2.3 ± 0.8 ^{b}	107 ± 54 ^{b}	0.19 ± 0.01	0.91 ± 0.06	1.08 ± 0.07

Mean ± SD are based on population means, which in turn are based on seed family means. Different letters indicate significant differences ($\alpha = 0.05$) among regions using Tukey's HSD test.

influenced by competition, whereas Central European and Alpine plants had thinner leaves when grown within competitive circumstances (data not shown). The competition × population interaction was significant for above-ground biomass, indicating differences among populations in competition ability. Different responses on competition were also found among seed families for flowering duration, flowering start and number of leaves, by testing the competition × seed family interaction (Table 12.2).
The competition ability, the relative response to competition in the above-ground biomass, averaged at 0.84 ± 0.11 (Table 12.4). The coefficient of variation assessed for competition ability among regions was smaller (CV = 0.178) compared to the relative variation within region (CV = 0.398). A correlation between competition ability and genetic diversity (H_E) was not found.

Table 12.4: Competition ability means ± SD per region (SC: Scandinavia, NL: The Netherlands, CE: Central Europe, AL: European Alps) and per population (Pop. codes see table 1), measured with above-ground biomass of *Campanula rotundifolia* in a common garden experiment with *Bromus erectus* as competitor.

Region	Pop.code	Competition ability
SC		0.96 ± 0.14
	SC_1	0.87 ± 0.00
	SC_2	0.89 ± 0.43
	SC_3	1.24 ± 0.20
	SC_4	0.89 ± 0.24
	SC_5	0.93 ± 0.15
NL		0.88 ± 0.16
	NL_1	0.79 ± 0.11
	NL_2	1.17 ± 0.61
	NL_3	0.90 ± 0.30
	NL_4	0.84 ± 0.27
	NL_5	0.69 ± 0.17
CE		0.71 ± 0.11
	CE_1	0.72 ± 0.17
	CE_2	0.92 ± 0.13
	CE_3	0.71 ± 0.16
	CE_4	0.60 ± 0.09
	CE_5	0.62 ± 0.15
AL		0.80 ± 0.09
	AL_1	0.89 ± 0.37
	AL_2	0.64 ± 0.24
	AL_3	0.86 ± 0.09
	AL_4	0.79 ± 0.49

Molecular diversity

AMOVA tests attributed 10% of the overall molecular variation among regions, 27% to among-population component within regions and the remaining 63% of the molecular variation appeared within populations (Table 12.5). STRUCTURE analysis and an UPGMA dendrogram did not show clear patterns in regional molecular differentiation (data not shown). Isolation by distance was absent (Mantel test between genetic and geographic distances; $P = 0.434$) and therefore could not explain regional molecular differentiation.
The allelic richness (N_A) was similar among all regions with the exception of the Dutch plants, showing a significantly lower value. The high variability of the Dutch allelic richness originated partly from an extremely low N_A in the population NL_1 (Anloo; $N_A = 58.8$). The highest genetic diversity (expected heterozygosity, H_E) was found for the Alpine populations, whereas the Dutch populations had the lowest value (Table 12.6).

Table 12.5: Competition ability means ± SD per region (SC: Scandinavia, NL: The Netherlands, CE: Central Europe, AL: European Alps) and per population (Pop. codes see table 12.1), measured with above-ground biomass of *Campanula rotundifolia* in a common garden experiment with *Bromus erectus* as competitor.

Source of variation	d.f.	% of variation
Among all regions	3	10
Within regions	152	27
Within populations	12	63
Among SC populatoins	3	20
Within SC populations	48	80
Among NL populations	3	37
Within NL populations	48	63
Among CE populations	3	28
Within CE populations	48	72
Among AL populations	3	29
Within AL populations	48	71

Table 12.6: Molecular statistics per region (SC: Scandinavia, NL: The Netherlands, CE: Central Europe, AL: European Alps) in *Campanula rotundifolia*.

Region	N_A	H_E
SC	84 ± 3	0.184 ± 0.02
NL	74 ± 6	0.164 ± 0.03
CE	88 ± 4	0.183 ± 0.02
AL	85 ± 5	0.238 ± 0.02

Per region 4 populations × 13 individuals × 27 loci were used to measure mean ± SD of allelic richness across all loci (N_A) and Nei's expected heterozygosity (H_E).

Heritability and Q_{ST}–G'_{ST} Comparison

The heritability of the continuous traits throughout all populations ranged between 0.40 and 0.64 (Table 12.7). The Q_{ST} value of above-ground biomass and flower shape exceeded G'_{ST}, indicating an influence of diversifying selection. All other traits had lower Q_{ST} values than G'_{ST}, indicating that 10 out of 12 traits are influenced by unifying selection. No traits showed significant overlap at α=0.05 (t-tests) of the Q_{ST} and the G'_{ST} value, and selection therefore caused at least part of the observed differentiation in phenotypic traits.

Table 12.7: Heritability and Q_{ST}-G'_{ST} comparisons for 12 phenotypic traits of *Campanula rotundifolia*, using common garden experiments and RAPDs analysis.

	h^2	Q_{ST}	Q_{ST} vs G'_{ST}
Above-ground Biomass	0.469 ± 0.217	0.513 ± 0.060	Diversifying
Number of capsules	0.494 ± 0.200	0.263 ± 0.086	Unifying
Flowering start	0.490 ± 0.262	0.294 ± 0.051	Unifying
Flowering duration	0.553 ± 0.230	0.019 ± 0.010	Unifying
Number of leaves	0.561 ± 0.205	0.040 ± 0.009	Unifying
Number of stems	0.404 ± 0.200	0.246 ± 0.042	Unifying
Stem length	0.516 ± 0.210	0.292 ± 0.034	Unifying
Leaf thickness	0.499 ± 0.206	0.064 ± 0.013	Unifying
Leaf shape	0.421 ± 0.278	0.193 ± 0.035	Unifying
Flower shape	0.635 ± 0.286	0.891 ± 0.238	Diversifying

Mean ± SD for narrow-sense heritability (h^2) and quantitative differentiation (Q_{ST}) and the deviation of Q_{ST} from G'_{ST} (= 0.415 ± 0.011). All comparisons were significant at $P < 0.001$

Discussion

The common garden experiment indicated regional genetic differentiation in the investigated phenotypic traits of *C. rotundifolia* across regions in Europe. In line with our hypotheses, some phenotypic traits, notably flowering phenology, showed a differentiation pattern along the latitudinal and altitudinal regions, whereas other traits, such as competition ability, had a more heterogeneous pattern of differentiation among the investigated regions and populations.

Differentiation in flowering phenology

Phenological traits were expected to vary clinally with latitude and altitude due to an association of the phenology with length of the growing season (Turesson 1930; Kalisz and Wardle 1994; Li et al. 1998). In line with this expectation the phenology of *C. rotundifolia* in the common garden revealed a genetically based pattern with latitude and altitude, viz. a decrease in flowering duration with increasing latitude and altitude as well as an advanced flowering start in high altitude populations and a delayed flowering start in high latitude populations. The shorter growing season at high latitude and altitude may force plant populations to shorten their flowering period in order to complete their reproductive cycle in time (Weber and Schmid 1998). Similar adaptation of flowering duration along a latitudinal gradient has been observed by Olsson and Ågren (2002) in *Lythrum salicaria*. Clausen, Keck and Hiesey (1947) showed the same correlation associated with an altitu-

dinal gradient for *Potentilla glandulosa*.
In order to finish the reproductive cycle in time and to optimally exploit the vegetation period, plants have to start flowering earlier in a short vegetation period compared to plants living in a longer season (Makrodimos et al. 2008). In line with this hypothesis, the Alpine populations of *C. rotundifolia* flowered earlier than the other populations in the common garden. The same selection for early flowering was expected for the high latitude populations, but the Scandinavian plants showed a comparatively delayed start of flowering. It is known that the environmental cue for flowering initiation is temperature or photoperiodism, or even an interaction between both factors (Keller and Körner 2003) It is not obvious whether the flowering response in the common garden of *C. rotundifolia* was sensitive to photoperiodism, temperature or both. By growing the populations in a common garden in Basel, the temperature conditions as well as the photoperiodic regime had been changed for foreign plants. The delayed flowering of the Scandinavian plants could be explained by assuming sesitiveness to photoperiodicity and by considering the increase of light hours per day towards high latitude. The Scandinavian plants would then have had more time under the specific photoperiodic regime of Basel until the threshold of light hours per day to initiate flowering was reached.
A study with nine *Campanula* species on Mt Olympos (Blionis et al. 2001) contradicts the altitudinal differentiation found in phenology in our common garden experiment. That study found a delayed flowering (3 d for every 100 m) with increasing altitude and a mid-altitude peak (ca. 38 d) for flowering duration. However, it is difficult to interpret this discrepancy of results because their observations were made on a range of species in natural field populations and not on plants grown in a common garden. Moreover, it is doubtful whether the Alps are comparable with the mountains in Greece from climatological perspectives.

Differentiation in growth and morphological traits

Above-ground biomass and number of capsules as well as the survival and flowering rates showed a decrease in fitness towards higher latitude and altitude of origin. This is probably caused by the discrepancy of climate and other environmental conditions in the plant's original site versus the common garden site. The home site populations, originating from locations with a maximum distance to the common garden of 20 km, showed the best performance. Since the summer temperature decreases significantly with altitude and since precipitation decreases across our regions northwards, the climate at the common garden site was probably too warm and too dry for the Alpine populations and too warm and too wet for the Scandinavian and the Dutch populations. Therefore, the variation observed in fitness traits of the Scandinavian, Dutch and Alpine population arose probably from a maladaptation to the common garden conditions and indicates that biomass and capsule production are adapted to the climatic conditions of the original sites.
The number of leaves increased with increasing altitude of origin. In contrast, the length of flowering stems decreased towards higher latitude and altitude, and the Scandinavian populations carried less flowering stems. This indicates that the plants originating from the colder biomes grew slower in the common garden. However, Scandinavian plants had comparably small stem lengths, but it is difficult to explain this observation as adaptation to climatic conditions. Scandinavian plants also bear more leaves compared to individuals from lower latitudes, but this is an effect of phenology since plants from lower latitudes were already flowering at the time of measurement, and their leaves had therefore already started to die back. Similar latitudinal patterns in growth and phenology have been observed in *Arabidopsis thaliana* (Li et al. 1998) and *Lythrium salicaria* (Olsson and Ågren 2002).
Flower shape (length/width) showed a weak regional differentiation (Tables 12.2 and 12.3). This observation could indicate that the flower dimensions are adapted to dimensions of various bee species, the most frequent pollinators. In contrast to these observations, stable flower shape across altitude and latitude has been observed before in *Campanula* species and other taxa (Blionis et al. 2001; Olsson and Ågren 2002; Fabbro and Körner 2004).
Alpine plants generally produce smaller and

thicker / denser leaves than lowland plants (Körner et al. 1989; Körner 2003). This mechanism is thought to optimize the efficiency of the photosynthetic tissue activity and the competition ability in colder biomes (Körner et al. 1983). In our experiment, the observed variation of leaf shape in the common garden contradicted this general trend, which could be due to phenotypic plasticity to common garden conditions. A strong plastic response is well known from many Alpine plants, for instance in transplantation studies to a different altitude (Scheepens et al. 2010a), and is likely an adjustment to temperature (Körner 2003).
The biomass and capsule production, initial flowering and most of the morphological traits correlated with latitude and altitude of origin as well as with climate variables of the sites of origin. This suggests that these traits are most probably adapted to climatic clines. However, the results could change depending on site conditions, i.e. using more than one transplant site could strengthen this conclusion. Reciprocal transplantation experiments would be superior to assess the degree of regional or local adaptation along a climatic cline (Rice and Mack 1991) since transplanted plants experience natural conditions.

Response to Competition

Plants generally showed a negative trend in the investigated phenotypic traits when grown with competitors in the common garden. For start of flowering, however, no significant competition effect throughout the regions was found, indicating that flowering is synchronised and largely independent of growth status (Heide et al. 1985). In spite of the increased survival rate of the plants that grew with competition, they obviously showed a decrease in biomass and capsule production. We speculate that *Bromus erectus* protected the plants from drought by shading, but affected growth negatively through competition for nutrients and light. The study of Pluess and Stöcklin (2005) similarly revealed a negative effect on reproduction and plant size in *Geum reptans* in a greenhouse experiment with *Poa alpina* as competitor.
The number and length of the stems and primarily the capsule production decreased significantly in response on competition. Partzsche and Bachmann (2010) found a similar strong decrease in fecundity in *Campanula glomerata* in a greenhouse experiment with two competitive grass species. These findings correspond to those of a study by Fréville and co-workers (2005), which attributed a stronger competitive effect to fecundity than to growth responses.
The capsule production and the thickness of leaves were differentially affected by competition depending on the region of origin (Region × Competition; Table 12.2), but variability within regions was large for number of capsules (Fig. 12.2d, Table 12.3). The effect of competition on biomass and flowering duration differed strongly among populations within regions (Fig 12.2a and c; Table 12.4), indicating local adaptation of competition ability. For flowering duration this suggests that, aside from adaptation to regional climate, populations are likely to be differentially adapted to more local environmental variability within regions, which is in line with our hypothesis.
A correlation between competition ability and genetic diversity was not found. Such a relationship has been found in a greenhouse study with *Scabiosa columbaria* and *Bromus erectus* as competitor where *Scabiosa* populations with a low genetic diversity correlated with a low competition ability (Pluess and Stöcklin 2004), indicating the adverse effects of loss of genetic diversity on fitness traits.

Molecular diversity

Molecular differentiation in neutral markers suggested a regional genetic structure in *C. rotundifolia*, since regions explained 10% of the variation. However explained variation was almost three times higher within regions than among regions. Isolation by distance, usually present in molecular marker studies at this scale (e.g. Hardy and Vekemans 1999; Kuss et al. 2008; Ramirez-Valiente et al. 2009), was absent in this study, which could be related to the strong isolation of populations in the landscape, which disrupts gene flow (Young et al. 1996).
The Dutch populations showed the lowest value in allelic richness, reaching the lowest value in the population Anloo – a small and isolated population. The Dutch populations also showed

the lowest genetic diversity. This may correlate with lower fitness and an increased risk of local extinction through an inability to adapt to climate change (Young et al. 1996), but empirical evidence for such relationships is still scarce (but see Pluess and Stöcklin 2004). Although competitive ability, an important fitness component, was not related with genetic diversity, Dutch populations may currently be declining in genetic diversity potentially bringing adverse effects in the future. The distribution of *C. rotundifolia* in the Netherlands is shrinking rapidly over the last decades (e.g. *Floron Nieuws* 2007), which is likely the result of habitat destruction and fragmentation, which leads to reduced gene flow (Young et al. 1996) and potentially causing a loss in genetic diversity.

In contrast, the Alpine populations, which were of similar size as Dutch populations but which are instead naturally fragmented, showed the highest genetic diversity. This has been observed before in other Alpine plant species (Stöcklin et al. 2009) and it could be speculated that the species evolved phenotypic traits related to seed and pollen dispersal in such a way that it retains sufficient genetic diversity and gene flow in a strongly heterogeneous landscape (Kuss et al. 2008). In the case of *C. rotundifolia*, this increased genetic diversity in the Alps could also be due to introgression of genetic material from related species. Additionally, molecular data should be treated with caution since ploidy levels vary in *C. rotundifolia*. The prevailing chromosome numbers are 2n = 34 and 2n = 68 (Shetler 1982), which could strongly affect the allelic richness (Table 12.6). However, since the lowest allelic richness in a population was 59 (Anloo) and all other populations ranged from 71–97 alleles (data not shown at population level), it suggests that all sampled populations have equal chromosome numbers.

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Heritability and past selection pressure

The observed heritability values indicated that the phenotypic variance was due to roughly equal proportions of genetic and environmental effects. Number of stems and leaf shape showed comparably low values of h^2, indicating that environmental conditions acted strongly on those traits, whereas flower shape, flowering duration and number of leaves had relatively high heritability, which can be expected for flower shape, which needs to be rather constant for pollinators, as well as for flowering duration which needs to be adapted to climatic conditions.

Unifying selection ($Q_{ST} < G'_{ST}$) caused the observed patterns for most traits, except for above-ground biomass and flower shape, for which diversifying selection ($Q_{ST} > G'_{ST}$) was indicated. Plants may need to decrease above-ground biomass in favour of root growth under colder conditions (Körner and Renhardt 1987) and genetic adaptation to the respective climates in this study may therefore have occurred in this trait. The reason why biomass experienced diversifying selection, as opposed to unifying selection, could be due to the polygenic complexity of this trait (Scheepens et al. 2010b). In environments where above-ground biomass should be altered, unifying selection on the available variability in this trait may be insufficient due to its slow neutral differentiation; diversifying selection is needed to adapt biomass to its environment. Though flower shape has been reported as being constant and adapted to

pollinator size (Olsson and Ågren 2002), we can accept a similar reasoning as for biomass, since across the climatic clines in our study pollinators, even of the same species, may differ in size due to climatic influence on growth of insects (the temperature-size rule; Atkinson 1996).

Conclusions

The phenology of *C. rotundifolia* showed clinal patterns across regions in Europe. Alpine plants showed an advanced start of flowering, indicating a response to the temperature regime in the common garden, whereas the Scandinavian plants delayed their start of flowering, possibly due to the discrepancy in the photoperiodic regime. The flowering duration indicated an adaptation towards a shorter growing season with increasing latitude and altitude of the sampled regions. Two important fitness traits – biomass and capsule production – also showed a clinal decrease with higher latitude and altitude, but this is likely to be a common garden effect. A strong heritable component (mean ± SD: 0.496 ± 0.068) was generally found in all phenotypic traits. Q_{ST}–G'_{ST} comparison indicated that past selection pressure acted on all phenotypic traits, either diversifying for above-ground biomass and flower shape, or unifying for the other traits. In conclusion, the results suggest that start and duration of flowering, the number of capsules and above-ground biomass of *C. rotundifolia* vary among regions. These traits are therefore likely adapted to environmental clines. The substantial heritability in these traits indicates that they have potential to adapt to future climate change. Morphological traits also showed regional differentiation, but this was not obviously related to climatic clines. Moreover, competition ability showed strong within-region variability and may be adapted to local conditions variable at this within-region scale. The RAPD study revealed a considerable regional component, but a clear spatial structure was not apparent. Dutch populations had lower genetic diversity, which is in line with the decrease in distribution of *C. rotundifolia* in The Netherlands. This result may therefore indicate a higher chance of extinction for the Dutch populations, although our results indicated that competitive ability, an important fitness trait, is currently not yet affected.

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Appendix

Table 12.8: Pearson correlation coefficients of phenotypic traits with geographic and environmental variables

			Latitude		Altitude	
	Latitude	Altitude	T_{mean}	Prec	T_{mean}	Prec
Above-ground biomass	-0.184 ***	-0.085 *	0.313 ***	0.377 ***	0.368 ***	-0.403***
Number of capsules	-0.155 ***	-0.063	0.163 ***	0.321 ***	0.272 ***	-0.264***
Flowering start	0.254 ***	-0.216 ***	-0.034	-0.307 ***	0.201 ***	-0.170***
Flowering duration	-0.032	-0.065	0.085	0.100 *	0.057	-0.079
Number of leaves	0.157 ***	-0.058	-0.092 *	-0.227***	-0.051	0.141 **
Number of stems	-0.249 ***	0.064	0.222 ***	0.364 ***	0.145 **	-0.120 *
Stem length	-0.334 ***	0.129 ***	0.194 ***	0.494 ***	0.066	-0.099 *
Leaf thickness	- 0.109 **	0.137 ***	0.042	0.094 *	0.033	-0.182 ***
Leaf shape	0.153 ***	-0.116 **	-0.102 *	-0.215 ***	0.003	0.150 *
Flower shape	0.192 *	-0.063	0.023	-0.277 ***	-0.122	-0.044
Competition	-0.057	0.051	0.054	0.035	0.015	-0.029 *

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Chapter 13

General Discussion

General Discussion

Summary

The aim of this thesis was to understand how a plant species' evolution towards its current state is affected by neutral genetic drift and historical as well as more recent environmental influences acting at different spatial scales. Based on this aim, several main questions were formulated in the General Introduction (**Ch. 1**) and these were addressed in the subsequent chapters (**Ch. 2-12**). I will now shortly summarise what we have learned from these studies with respect to these questions. The question *whether glacial history affected phenotypic differentiation in widespread alpine plants* has been answered positively. We showed that phenotypic differentiation in various traits of *Campanula thyrsoides*, *C. barbata* and *Geum reptans* mirrors phylogeographic divergence which is related with glacial survival in separate refugia (**Ch. 3, 4, 5, 6, 10, 11**). Even when accounting for distance-related differentiation, *Campanula thyrsoides* and *C. barbata* retained differentiation among phylogeographic regions. This finding is novel; although many studies investigated and compared molecular with phenotypic differentiation, specifically to infer past selection pressures (Leinonen et al. 2008), we showed that such phenotypic differentiation is a consequence of glacial history.

The follow-up question *whether any such glacial history-related phenotypic differentiation is the result of adaptation or neutral genetic drift* proved more difficult to answer, and the results are suggestive rather than confirmative. Correlation of trait values with environmental conditions at sites of origin could show current adaptation, which could be in line with small-scale heterogeneous variability in the landscape. However, it could also mirror regional environmental differences to which phylogeographic lineages could have adapted, whether recently or in times of glaciations. In the former case, putative adaptation to heterogeneous conditions would be relatively recent and would originate from post-glacial times. The latter case, however, would be more suggestive of adaptation during glaciation, although it is not a definite proof.

An example of potential glacial history-related regional adaptation was most clearly visible for *C. thyrsoides*, which showed a strikingly different phenology and morphology in the Southeastern Alps compared to regions to the west, which is in line with the differences in altitude and climatic conditions between these regions (**Ch. 4, 5, 6**). We argued that the contrasting climatic conditions during glacial survival caused strong differential adaptation that affected its current distribution and even led to allopatric subspeciation. Related to the aim of this thesis to understand past versus current processes which affect evolution, the results from **Chapters 2, 4, 6, 11 and 12** suggest that selection is pervasive in nature since Q_{ST}–F_{ST} comparisons indicated that practically all investigated traits experienced past selection pressures, whether unifying or diversifying. However, adaptation and genetic drift are not mutually exclusive and a large part of the differentiation in measured traits can still be due to neutral processes. Although it is possible to answer whether or not selection acted on a trait, there is no method that can exclude neutral genetic drift, and it is methodologically difficult to assess the proportion of neutral differentiation that affected the observed trait differentiation. I will further discuss methodological difficulties concerning neutral differentiation versus adaptation in the Outlook (see below).

Chapters 7, 8 and 9 gave insight into the second set of questions relating to the monocarpic perennial *C. thyrsoides*, (i) *how seed and pollen dispersal and the resulting genetic diversity are structured in time and space*, (ii) *what this tells us about the colonisation history and future*, and (iii) *how the seeming contradiction between levels of within and among genetic diversity can be explained*. Former indications of considerable among-population differentiation and high within-population genetic diversity were reconfirmed for a group of populations on a small subalpine plateau (**Ch. 7**). Some of these pop-

ulations experienced genetic bottlenecks in the recent past, but gene flow among populations is probably high enough to overcome effects of drift. This high gene flow is probably not due to seed dispersal, since the strong seed dispersal limitation, as predicted from a dispersal model, was confirmed in a seed sowing experiment (**Ch. 9**). Indeed, a paternity analysis suggested that pollen flow is abundant among the populations on this mountain plateau, which connects these populations genetically. The putative contradiction of considerable among-population differentiation versus high genetic diversity within populations was solved by taking into account the effects of monocarpic perenniality on the population genetic structure. Monocarpic perenniality can lead to strong temporal retriction on gene dispersal since a large part of the genetic diversity is immobile (Vitalis et al. 2004). Nevertheless, this immobilisation of genetic diversity does not affect the high genetic diversity within populations, whereas the limited potential of pollen flow leads to genetic differentiation among populations.

Methodological limits

Scientific methods can be ingenious, but they have their limits too. Apart from limits in the extent that scientific methods can answer our questions, there is the enduring limit in time and financial resources. In this section I will shortly discuss a few issues concerning such limits and, if existing, their idealistic or practical alternatives.

Whether and to what extent adaptation or neutral genetic drift caused phenotypic differentiation during glacial survival in refugia is hard to conclude based on the current distribution of phenotypic differentiation ca. 135,000–700,000 years after the Quaternary glaciations presumably started to influence species. Several methods have been applied: (i) common garden experiments reveal phenotypic differentiation and are a first step in investigating whether there is genetically based variation which is not the result of environmental conditions of origin. However, differentiation observed in common garden experiments can at best be used to suggest patterns of adaptation in relation to the population's home environment (Clausen et al. 1948); (ii) we have already discussed the methodological limits of correlations between trait values observed in the common garden and environmental values at the sites of origin. These can suggest – but not prove (Kawecki and Ebert 2004) – adaptation to current conditions. Besides correlation of trait values and heterogeneous conditions, correlations may also suggest adaptation to regionally important environmental conditions and may in such a way be related to glacial history where different lineages recolonised particular climatic regions; finally, (iii) Q_{ST}–F_{ST} comparisons can reveal past selection pressure, but do not say anything about the time that selection took place.

So the methods are clearly limited in the extent that they can undoubtly reveal what part of the observed genetic differentiation is be due to adaptation to environmental conditions experienced during and after glacial survival. This limitation to prove adaptation leads, unwillingly, to a „search adaptation and you will find it“ approach (Gould and Lewontin 1979). The unidirectional approach, i.e. neutral differentiation in the absence of correlations or any sound explanation for the observed patterns on the one hand, and adaptation on the other hand) makes it difficult to prove neutral differentiation as a result of glacial history. It is therefore important to keep in mind that absence of evidence is not evidence of absence when a particular pattern cannot be explained by adaptation.

There are sound methods to prove adaptation. For instance, reciprocal transplantation experiments can prove local adaptation, but can in itself not be used to determine which environmental factors different populations are adapted to (Joshi et al. 2001; Kawecki and Ebert 2004). For this, either controlled experiments can be conducted in which a potentially influencing factor is varied and its effect on fitness is estimated, or reciprocal transplantation experiments can be conducted in sites with known variability in an environmental factor (e.g. Van der Wal et al. 2000; Santamaría et al. 2003). We have unanalysed data from our multiple common garden experiment (**Ch. 6**) which can be analysed in such a way.

To prove that at least part of the phenotypic differentiation is due to genetic drift, is more difficult. Perhaps Bayesian coalescent approaches

(Lascoux et al. 2003) or approaches similar to bioclimatic niche modelling (Yennon and Culham 2006) may be able to combine patterns of genetic and phenotypic divergence, giving insight not only in the species' genetic, but also in its phenotypic, history. Sudden, rapid changes in phenotypic divergence would then be suggestive for selection and continuous, slow changes would be suggestive of neutral differentiation.

Genetic versus plastic effects – The question how much of the observed trait variability is due to genetic and how much to plastic effects (**Ch. 6**) can only be answered while realising the boundaries of the experiment, which are flexible. If a larger elevational transplantation gradient was chosen, the contribution of plastic effects to the overall phenotypic variation would likely be greater. Theoretically there are two reference points determinable concerning the ratio of genetic versus plastic effects. Firstly, there exists an absolute and relative extent of genetic versus plastic effects measured as the most extreme points along a trait continuum. In order to determine these points, the maximum extent of phenotypic plasticity should be assessed by growing plants in multiple common gardens along the full (even unnatural) range of conditions of the environmental factor of interest. This should be done with genetic material from all existing populations in order to find the maximum genetic differentiation between two populations. Secondly, there exists an absolute and relative extent of genetic versus plastic effects measured as the naturally exhibited range of phenotypic instantiations. The method which can be applied here is similar to the one above, but now only the natural range of values for the environmental factor of interest is observed. The genetic effects could be, but is not necessarily, identical to those in the firstly discussed range of genetic effects, but the range in phenotypic plasticity is likely smaller. These proposed experiments are purely theoretical and can probably only be approximated. In relation to our experiment, the range in altitude of population origins was much larger (283–2266m a.s.l.) than the three altitudes covering the experimental sites (600–1235–1850m a.s.l.), which suggests that not even the natural, let alone the potential, range of plasticity has been observed in our experiments.

Outlook

The studies presented in this thesis naturally led to many follow-up questions. An open, and answerable, question is when the observed phylogeographic groups in different investigated species started to form. It is unclear whether the observed molecular differentiation in the studied species (*C. thyrsoides* – **Ch. 3**; *C. barbata* – Thiel-Egenter et al. 2011; *G. reptans* – **Ch. 11**) is the result of survival in refugia during the last Ice Age or during distribution changes over multiple glaciations. In order to date the split of the different regions in *C. thyrsoides*, chloroplast DNA is currently being sequenced and a molecular clock analysis will be applied to date the breaking into different lineages (Kadereit et al. 2004).
Another open question concerns the subspecies status of *C. thyrsoides* subsp. *thyrsoides* and subsp. *carniolica* (**Ch. 3, 5**). It is unknown whether the two subspecies can produce viable offspring, which is an important issue relating to the taxonomic and conservation status of this species, but it is also interesting to know whether glacial survival in refugia with contrasting climates could lead to speciation (**Ch. 3**). To investigate this issue, we planned to raise progeny of both subspecies to perform crossings between them.

Q_{ST}–F_{ST} comparisons – Comparing Q_{ST} with F_{ST} is now common practice to infer past selection pressures, but its theoretical basis as well as its justified application are still heavily debated (**Ch. 2**). There is a gap to fill among three disciplines here: (i) quantitative genetics, which provide Q_{ST}, (ii) population genetics, providing F_{ST} and (iii) molecular biologists, whose insight is needed to establish relationships between quantitative traits and their underlying coding genes (QTL; McKay and Latta 2002). Only at the genomic level is it possible to discover how both indices relate to each other mechanistically. Studies are needed that track the fates of neutral versus coding regions in the genome. It is also necessary to investigate the effect of trait complexity on the trait behaviour

under selection and drift (e.g. Morgan et al. 2005).

Conclusions

Using widespread plant species in the European Alps as model systems, I explored three biological elements in this thesis: (1) effects of neutral genetic drift, natural selection and phenotypic plasticity on phenotypic differentiation; (2) effects of glacial history, geography and climate on phenotypic differentiation and adaptation; (3) genetic structure and gene flow at small spatial scale. The aim was to understand how a plant species' evolution towards its current state is affected by historical as well as more recent environmental influences acting at different spatial scales.

The studies reported in this thesis showed that glacial history had strong effects on phenotypic differentiation, and that part of this differentiation is due to adaptation to past as well as current conditions, though neutral genetic drift may also have a substantial contribution to differentiation. In addition to drift and selection, it is probably due to strong spatial and temporal heterogeneity in environmental conditions in the European Alps that phenotypic plasticity evolved and forms an unignorable part of the functional phenotypic variability found in nature. Of all environmental variability in the Alps, those factors related to elevation are most pronounced, and many phenotypic traits have been found correlating with elevation. In populations across the Alps, molecular differentiation in *C. thyrsoides* was high and partly related to glacial history, but molecular differentiation was also found to be considerable at small spatial scales, which has been attributed to effects of the species' monocarpic perenniality on the among-population genetic structure.

I hope this thesis is a humble contribution to and support of Charles Darwin's conviction that *Natural Selection has been the main but not exclusive means of modification.* To speak with Albert Einstein, it takes time and effort to *look deep into nature*, but *then you will understand everything better.* Mao Tse-Tung wrote that *when you have investigated the problem thoroughly, you will know how to solve it*; so it is wise to take some time for such deep journeys of discovery, as Lao Tzu said about Nature, which *does not hurry, yet everything is accomplished.*

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Many friends at „home“ often asked me: „Why Switzerland?“ Against their expectations, I did not choose this project because of the mountains but because of the intriguing ecological and evolutionary questions one can answer in mountain systems. The Alps are indeed a laboratory, and I made much use of that fact the last three and a half years.

But more important was the fact that I found a superb supervisor after an afternoon's job interview that summer in 2007 – or I should rather say: after an interesting discussion on biology, philosophy, politics etc. Indeed, Jürg, my supervisor, receives the most grateful acknowledgement. The trust you had in me, and that I could have in you, created a very open and interactive atmosphere. I will always remember those instances that I came around for only a quick question, which turned out to become long discussions, whether on sampling designs, green politics, or Venice. I always experienced much freedom; freedom to follow my own interests and to plan most of the research myself, whether in its content or in timing. Though clear boundaries were given from the start by the SNF research proposal that was granted to Jürg, these never felt as limiting since they coincided with my own interests. Jürg, I have learned a lot from you, whether it concerns formulating biological questions, design of experiments, data analysis, or the communication of results via text and spoken word, but also concerning your personal view on biology (*each species has its own story*) and, indeed, your other interests in the world at large, trying to see clear through all its complexity.

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Lucienne came to our group in January 2008. As an Urner with Zeelandic roots, you were the reason I did not loose my own language. Though I soon recognised that I, too, had problems with germanisms in Dutch. More importantly, though, I thank you for the great time we had. We had much fun visiting Reinout in Vienna and experienced a nice but dangerous trip to Rumania for your fieldwork. I am also deeply grateful to all the help you gave me, whether in the field, in the office, or behind the wheel.

Although I worked in a small research group, a thesis is never the result of a single person. As the reader might have noticed, publications involve multiple persons, but beyond these, many people helped in one way or another, hopefully all of them being acknowledged in the resulting papers. One of the first whom I met on the workfloor were Guy and Edith from the Botanical Garden, who assisted me with growing literally thousands of plants in the greenhouse. Moreover, the common garden experiment at the Calanda Mountain in the summer of 2008 would not be possible without the help of Guy. It was the physically most tough work I can remember, to carry wood and bags of soil upslope to install our plant beds.

Veronica, „my“ first master student, worked in our research group under my direct supervision on her master thesis, starting in spring 2009. This was a great success as the experimental results were very interesting. It came to a nice text which will now soon be submitted for publication and which forms a great basis for your future career. A first big step you have taken recently by starting a PhD in Wageningen. Directly after my arrival in Basel, my colleagues noticed that I could speak some pidgin German, and so I was forced to speak and improve on it, especially very insistently by Martin B., for which I thank him. Additionally, I also acknowledge Martin's help with statistics and graphics in R – I still draw most of my graphics in R „by hand“.

Patrick and Hafdís Hanna were always there to answer my questions on the species that they, as my predecessors, know so well. I am glad that the collaboration with Patrick brought two nice publications. I also thank Andrea P. for the collaboration that led to another publication.

There were many people who assisted with fieldwork and in the laboratory. With the fear to forget some of them, I try listing them here: Nadja, Olivier B., Lucienne, Christine A., Stephan, Jasmin, Kai and Christine H., Bärbel, Judith, Katharina, Frau Just from Haldenstein for her friendly nature and the warm cup of tea on the cold early morning of fieldwork at Mt Calanda, Melissa for hosting me in Davos Clavadel, Miriam, Sonja.

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Christian, thanks to you for running the institute and for your ability to invoke interesting discussions, whether during the Monday morning seminars or during the coffee breaks. Thanks to Franziska G. and Esther for running the secretary of the institute and for always being there to help out. I also thank George for technical assistance in the greenhouse, and Erwin A. for the big logistical restructuring of the institute.

Fatou, thank you so much for your work, which includes so much more than cleaning the institute. We could laugh, we could dance, we could exchange our sorrows. You were always a strong support. On top of that, I will never forget your kind hospitality in your house in Dakar during me and Reinout's trip to your beautiful country.

I thank Irma for improving my German at the Sprachenzentrum and Erwin S. for teaching me to drive.

An important acknowledgement goes to the Döckelichäschtli and to those with whom I spent all those physically active games which are so necessary to bring back blood to the brain.

I also thank my teachers and supervisors from the Stedelijk Gymnasium and the Rijksuniversiteit Groningen for the excellent education, and Kuke and Jan for their letters of recommendation.

I also thank Eva F. It is a pity that there are differences and difficulties between us which proved hard to overcome. I wish you all the best for the future.

Not to forget, I thank my 2GB USB stick for outstanding service over the course of almost four years, and still going strong. It is funny to think that I always carried my thesis-in-progress with me in my pocket wherever I went.

Thanks go to the Open Source movement. I am grateful to Ubuntu-Linux, R and LaTeX, with which this thesis has been written. Though not open source, the Apple MacMini in the office also served me wonderfully.

Last but not least in this part of the acknowledgements, I sincerely thank my financial support without which everything would be different: (1) the Swiss National Science Foundation, project no. 3100AO-116785 to Jürg Stöcklin, (2) the Freiwillige Akademische Gesellschaft to me and (3) the Prins Bernhard Cultuurfonds to me for financing what became Veronica's master thesis and, hopefully, our first joint publication.

Thanks to those who supported me and my thesis indirectly

The above-mentioned freedom in timing when and when not to work on my thesis left room for unprecedented travelling and discovering of the world and the necessary distraction from work that comes with it. These travels included, for instance, longer stays at the many conference locations I have visited, meeting up with Reinout, Anneke and Collette for cycling in Luxembourg, visiting Dutch friends and family after the PopBio meeting in Nijmegen, and visiting Serinde after the BES meeting in Leeds. The Samos and Tenerife excursions can also be considered as botanically very interesting holidays.

But foremost I appreciated the well-spent time with Reinout – I named my EndNote reference list after you – on two botanical and cultural explorations in Western Africa. Our synergy is amazing. Thanks also go to Margherita. It was wonderful to explore your Vienna and the Hofburg with you, Reinout and Isabel.

Isabel meant a turning point for me in Basel, not only through your amazing person, but also since you were able to change the focus of my attention from nature outside of Basel towards the overload of culture in Basel. It was an insight to discover recently that the interstellar beauty of Guillaume Dufay's *Inclita stella maris*, which you sent me soon after we first met, represents in a special way our personalities: a canon in which the upper voices sing the same melody but at slightly different speeds.

José came back from fieldwork in Bolivia in January 2009. We had great movie nights and arabic nights and we did a course in Arabic together. It was through you that I got to know the great people from the Socinstrasse, who made me move out of my appartment to join them in spring 2010. A major turning point.

My time at the Socinstrasse has been just terrific. I thank all my housemates for the really great time in and outside the house, including Lupe, Urska, Daniel and Daniel who are more or less part of the community.

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Jule, thanks for the nice time in Giessen, Jena and München.

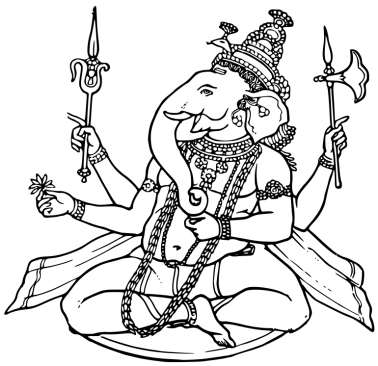
I thank the Universitätsorkester and their cellists, especially Hendrik (Bovistop!).

Meeting Leonardo was another great social turning point, as he reinvigorated my love for baroque music and made me try the viol after playing cello three semesters in the University Orchestra. Through Leo, a new world at the Schola Cantorum opened up for me. Not to forget saying that it was also with him that I had the most interesting conversations and discussions on all realms of life. To speak with Aldous Huxley, after silence, that which comes nearest to expressing the inexpressible is music. I wish to sincerely thank my musician friends Ozan (harpsichord), Leonardo, (viol), Sara (vocal chord, but we did not yet make music together), Eliane (viol), Helga (harpsichord, viol), Alice (Recorder), Silja (viol) and Basil (viol).

I am very grateful to Rebeka, my viol teacher. I hope to learn so much more from you. You are a reason for me to stay in Basel.

The Dutch team on the workfloor was strengthened by Thijs, who arrived from Wageningen in spring 2009. About the same time, Sietse joined the Hebelstrasse group, but I met you only much later due to the fractionated state of the various sub-institutes. Things turned cosy when we started our biweekly Dutch lunches at E9 together with Eelco from the Basel Institute on Governance. We soon banned talking about Switzerland from a Dutch perspective; instead, our most favourite topic became Dutch politics. Of course I should not forget to mention your counterparts: Ilja, Sjoukje and Sandra, respectively.

Finally, I thank my family and friends from The Netherlands for their enthousiasm, interest and support. I was often asked if I would return to The Netherlands afterwards, which I could interpret as a sign they missed me, like I missed them. I may have to disappoint here, since I live in an age and profession where country borders are vague. However, you have to know that you are and will always be close to my heart. My deepest acknowledgement goes to Rini, who supported me profoundly. I am also grateful to Coby, Fleur and Ann, Peet *in absentia*, Oma Gees, Oma Lien, Peter, Yvonne and Jeannette, Wiebe, Dikran and Ria, Ruud, Trienke, Kiki. I wish I could relay my thanks to Roland. Many thanks also go to my contemporaries at home, again with fear to forget people in this rush hour: Anne Marthe, Anneke, Wieke and Hugo, Martijn and Hendrike, Renske, Klaas, Maarten, Reinout and Margherita, Jurrian, Paultje, Mirka, Joost, Eelco, Serinde, Kirsten, Collette, Lemke, Mans, Rosa ... and the others.



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Curriculum Vitae

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Education

1988–1995 Primary school, Michaëlschool Leeuwarden (Waldorf), The Netherlands

1995–2001 Secondary school, Stedelijk Gymnasium Leeuwarden, The Netherlands

2001–2007 Master of Science in Biology, Rijksuniversiteit Groningen, The Netherlands, *Cum laude*
Master projects:

«Clonal structure of Elytrigia atherica – a study to reveal the growth strategy of a native invasive species» Supervision: Prof. J.P. Bakker and R.M. Veeneklaas

«Microbial community composition in arctic waters – a temporal and spatial study» Supervision: Prof. Dr. A.G.J. Buma and Dr. A.M.-T. Piquet

«Prehistoric vegetation reconstruction of the archaeological site of Swifterbant – a pilot study based on macro-remains» Supervision: Dr. R.M. Bekker.

2002–2007 Bachelor of Arts in Philosophy of Science, Rijksuniversiteit Groningen, The Netherlands

Bachelor thesis: «Structures in scientific and traditional knowledge systems – A comparison based on ecological knowledge» Supervision: Prof. Dr. T.A.F. Kuipers

since 2007 PhD candidate at the Botanical Institute, University of Basel, Switzerland.

Title of the NSF-funded Project: «How glacial history, selection and current gene flow affect alpine plants: Population differentiation, local adaptation and demography in a fragmented landscape» Doctoral supervisor: Prof. Dr. Jürg Stöcklin

Publications included in the thesis

Kuss P, Armbruster GFJ, Ægisdóttir HH, **Scheepens JF**, Stöcklin J (2011) Spatial genetic structure of Campanula thyrsoides across the European Alps: indications for glaciation-driven allopatric subspeciation. *Perspectives in Plant Ecology, Evolution and Systematics*, doi: 10.1016/j.ppees.2011.02.003

Scheepens JF, Kuss P, Stöcklin J (2011) Differentiation in morphology and flowering phenology between two Campanula thyrsoides L. subspecies. *Alpine Botany*, 121(1), 37–47

Scheepens JF, Stöcklin J, Pluess AR (2010) Unifying selection acts on competitive ability and relative growth rate in Scabiosa columbaria. *Basic and Applied Ecology*, 11: 612–618

Scheepens JF, Frei ES, Stöcklin J (2010) Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to different altitudes. *Oecologia*, 164(1), 141–150

Publications not included in the thesis

Bruun HH, **Scheepens JF**, Tyler T (2007) An allozyme study of sexual and vegetative regeneration in Hieracium pilosella L. *Canadian Journal of Botany*, 85(1), 10-15

Scheepens JF, Veeneklaas RM, Van de Zande L, Bakker JP (2007) Clonal structure of Elytrigia atherica along different successional stages of a salt marsh. *Molecular Ecology*, 16, 1115-1124

Piquet AM-T, **Scheepens JF**, Bolhuis H, Wiencke C, Buma AGJ (2010) Variability of protistan and bacterial communities in two Arctic fjords (Spitsbergen). *Polar Biology*, 33: 1521-1536

Teaching experience

2006 Assistent for first-year undergraduate course *Statistiek*, 2 weeks, L.P.W.G.M. van de Zande, University of Groningen, The Netherlands

2007 Assistent for first-year undergraduate course *Biomathematica*, 3 weeks, F.J. Weissing, University of Groningen, The Netherlands

2008 Assistent for Blockkurs *Ökologie – Teil Pflanzenökologie*, 1 week, Ch. Körner i.a., Botanical Institute, University of Basel, Switzerland, Field location:St. Bauzille de Putois, Cévennes, France

2008-2010 Two-hour lecture on Q_{ST}–F_{ST} analysis in the course *Pflanzenökologie und Populationbiologie*, Ch. Körner and J. Stöcklin, Botanical Institute, University of Basel, Switzerland

Courses attended during the thesis

Planung und statistische Auswertung von Experimenten in der Ökologie – Dr. Pascal Niklaus, 2007

Pflanzenökologischer Feldkurs im Ausland mit Vorbereitungsseminar (Mediterrane Ökosysteme, Griechenland) – Prof. Dr. Christian Körner and Prof. Dr. Jürg Stöcklin, 2008

Contemporary Applied Statistics for Ecology – Ass. Prof. Dr. Andy Hector, 2009

Pflanzenökologischer Feldkurs im Ausland mit Vorbereitungsseminar (subtropische Ökosysteme, Kanaren) – Prof. Dr. Jürg Stöcklin and Dr. Martin Bader, 2009

Conferences and meetings attended during the thesis

(with authors and title of contribution)

Biology08, Lausanne, Switzerland, January 2008. Without contribution.

GfÖ Plant Population Biology Meeting, Luxembourg, Luxembourg, May 2008.
Scheepens JF, Pluess AR, Stöcklin J. *The role of selection in differentiation of* Scabiosa columbaria *populations in the Swiss Jura.* Oral presentation.

EURECO-GfÖ Jahrestagung, Leipzig, Germany, September 2008.
Scheepens JF, Armbruster GFJ, Ægisdóttir HH, Kuss P, Stöcklin J. *Spatial genetic structure of* Campanula thyrsoides *reveals four evolutionary units possibly mirroring postglacial recolonization.* Oral presentation.

Symposium der Schweizerischen Botanischen Gesellschaft und der Basler Botanischen Gesellschaft zum Darwin-Jahr, Basel, Switzerland, September 2008. Without contribution.

Lab visit at Bangor University, Molecular Ecology and Fisheries Genetics Laboratory, Bangor, Wales, UK. October 2008.
Scheepens JF, Armbruster GFJ, Ægisdóttir HH, Kuss P, Stöcklin J. *Spatial genetic structure of* Campanula thyrsoides *reveals four evolutionary units possibly mirroring postglacial recolonization.* Oral presentation.

Biology09, Bern, Switzerland. February 2009.
Scheepens JF, Frei ES, Stöcklin J. *Separating genotypic and environmental variation in specific leaf area in a widespread Alpine plant.* Oral presentation.

GfÖ Plant Population Biology meeting, Bern, Switzerland, May 2009.
Scheepens JF, Frei ES, Stöcklin J. *Genotypic and environmental variation in specific leaf area in a widespread Alpine plant after transplantation to different altitudes.* Oral presentation.

ProClim 11th Swiss Global Change Day, Bern, Switzerland, April 2010. Without contribution.

GfÖ Plant Population Biology, Nijmegen, The Netherlands, May 2010.
Scheepens JF, Frei ES, Stöcklin J. *Regional differentiation in life history and susceptibility to grazing in a widespread alpine monocarp.* Oral presentation.

GMBA-DIVERSITAS meeting, Chandolin, Switzerland, July 2010.
Scheepens JF, Frei ES, Stöcklin J. *Regional differentiation in life history and susceptibility to grazing in a widespread Alpine monocarp.* Oral presentation.

SymBioSE biology students meeting, Eskisehir, Turkey, August 2010.
Scheepens JF, Frei ES, Stöcklin J. *The influence of glacial history on the evolution of alpine plants.* Oral presentation.

GfÖ Jahrestagung, Giessen, Germany, August–September 2010.
Scheepens, JF, Frei ES, Stöcklin J. *Regional differentiation in life history and susceptibility to grazing in a widespread alpine monocarp.* Oral presentation.

British Ecological Society Annual Meeting, Leeds, UK, September 2010.
Scheepens JF, Armbruster GFJ, Ægisdóttir HH, Kuss P, Stöcklin J. *Regional differentiation in phenotypic traits and plasticity in an Alpine monocarp reflects its molecular genetic structure.* Oral presentation.

Schweizer Botanische Gesellschaft – Jahresversammlung und Symposium „Alpine Botanik“, Bern, Switzerland, September 2010.
Scheepens JF, Kuss P, Stöcklin J. *Morphologische und phenologische Differenzierung zwischen zwei* Campanula thyrsoides *Unterarten.* Oral presentation.

Cracks in the Concrete Jungle–New Perspectives on Urban Ecology, Berlin, Germany, October 2010. Without contribution.

Professional memberships

BES British Ecological Society

FAG Freiwillige Akademische Gesellschaft

GfÖ Gesellschaft für Ökologie

NIBI Nederlands Instituut voor Biologie

SBG Schweizerische Botanische Gesellschaft

Funding secured

Prins Bernhard Cultuurfonds

Freiwillige Akademische Gesellschaft

Theses pertaining to the Dissertation entitled

Phenotypic divergence in widespread plants: genetic drift, selection and plasticity

by

Johannes Fredericus Scheepens

1. Glacial history not only caused neutral genetic differentiation (Ch. 3) but also phenotypic divergence in widespread Alpine plants (Ch. 4, 5, 6, 10, 11).
2. Even in a world without selection, where you happen to be will most likely affect the evolution of your offspring (Ch. 3, 4, 5, 6, 10, 11).
3. Substantial genetic differentiation among populations does not necessarily contradict strong gene flow among those populations when it concerns monocarpic perennial plants (Ch. 8).
4. Scientific methods can be like a wolf in sheep's clothes – use them at your own risk (Ch. 2).
5. The mathematician says that *Nature always tends to act in the simplest way* (Johann Bernoulli); his profession, however, *would certainly have not come into existence if one had known from the beginning that there was in nature no exactly straight line, no actual circle, no absolute magnitude* (Friedrich Nietzsche – Menschliches, Allzumenschliches).
6. Although the custom to write species names in Latin is retained till this day, its grammatical implications are usually ignored. For instance, *Campanula thyrsoides*, although feminine, is not to be referred to as she or one is willing to be seen as a joker.
7. One must consider the semantic implications of language used in describing dispersal of plants, animals and people. The understanding of the term immigrant depends on the scale of perception (Paraphrased from Esther Chang; Ch. 8).
8. Wisdom becomes knowledge when it becomes your personal experience (Yogi Tea label proverb).
9. The fittest, identified by their better survivorship, survive better (R.H. Peters – Critique for Ecology).
10. The number of theses that nobody can deny is infinite (Willem Frederik Hermans – Nooit meer slapen).
11. The identical names of some famous Anglo-Saxon philosophers and Baroque composers is a result of sheer chance ($P > 0.05$).
12. Met Sint Juttemis promoveren zou leuk geweest zijn, maar jammer genoeg is dat een feestdag.
13. This dissertation is ready ($P < 0.05$).