Plant reproduction in the alpine landscape

Reproductive ecology, genetic diversity and gene flow of the rare monocarpic *Campanula thyrsoides* in the Swiss Alps

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Prof. Dr. Jürg Stöcklin
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Prof. Dr. Hans-Peter Hauri
Dekan

Mig hefur leingi lángað til að verða flökkukona...
Það hlýtur að vera gaman að sofa í lýngbrekkum hjá nýbornum ám.

Halldór Laxness. Íslandsklukkan. Hið ljósa man, 2. kafli.

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Chapter 1

General introduction

Alpine ecosystems and fragmentation

Habitat fragmentation, i.e. the process of large continuous habitats being subdivided into several smaller spatially isolated remnants, is a serious threat to biodiversity in many terrestrial ecosystems and the root cause of many conservation problems (Young et al. 1996; Frankham et al. 2002). Although discussion on habitat fragmentation, habitat destruction and species extinction has largely been confined to the anthropogenic destruction of formally continuous natural habitats, such as temperate grasslands and tropical rainforests (see e.g. Turner et al. 1996; Debinski & Holt 2000), the fragmentation of other ecosystem types should not be disregarded. Alpine ecosystems are commonly described as naturally strongly fragmented, with many microhabitats, created by the pronounced environmental gradients and the heterogeneous topography of alpine landscapes. These landscape characteristics can greatly affect the life of alpine plants (Körner 2003).

We are living in an era of global change, not only in the form of increasing habitat fragmentation and destruction, but also as a result of climatic warming and land use changes, which can have serious consequences for the world's ecosystems (Saunders et al. 1991; Theurillat & Guisan 2001; Walther et al. 2002; Körner 2003). In the alpine life zone, changes in land use, both in the form of more intense pasturing or, the reverse, abandonment of former, traditionally pastured alpine terrain are considered to be the greatest risk to the loss of alpine biodiversity (Körner 2001, 2003). Moreover, alpine ecosystems have repeatedly been put in the spotlight due to their vulnerability to global warming, with its potential impact on snow cover and permafrost as well as upward migration of species and vegetation shifts, which could lower available surface area and affect the plants' growing conditions (Grabherr et al. 1994; Theurillat & Guisan 2001). With regard to global warming, knowledge on the genetic variability in alpine plants has become particularly important since high genetic diversity is considered essential for plants to keep pace with the changing environment (Booy et al. 2000; Theurillat & Guisan 2001; Till-Bottraud & Gaudeul 2002).

This thesis comprehensively studies the effects of natural fragmentation and patchiness on the life of isolated plant populations in the fragmented alpine landscape. The focus is specifically on its effects on plant reproduction, such as mating system

and inbreeding depression, as well as on the genetic variability and gene flow within and among alpine plant populations.

Plant reproduction in the alpine landscape

Although, many alpine plant species are long-lived and rely on clonal growth for reproduction (Billings & Mooney 1968; Bliss 1971; Körner 2001), the importance of sexual reproduction, should not be understated, above all in the case of plants living in ever-changing and fragmented environments. Unlike clonal growth, sexual reproduction can by risky, especially for species that are unable to self-pollinate, but it is nevertheless important for alpine plants in order to retain genetic diversity and to develop ecotypic differentiation of traits (Körner 2001).

Sexual reproduction in alpine plants is dominated by outcrossing although the majority of alpine plants are also able to self-pollinate (Körner 2001, 2003). That is not surprising bearing in mind that according to Baker's law, self-compatibility should be favoured in plant species living in isolated populations in a fragmented landscape in order to ensure sexual reproduction if pollination fails (Baker 1955, 1967).

Despite self-compatibility being frequent among plants, including alpine plants, many monoecious and hermaphroditic plant species have mechanisms that promote outcrossing and prevent self-fertilization, such as dichogamy or self-incompatibility system (Richards 1997; Byers & Waller 1999). Dichogamy promotes outcrossing by seperating pollen shedding and stigma receptivity in time. It doesn't however guarantee, outcrossing since multiflowered plants might be self-pollinated with pollen from other flowers on the same plants (geitonogamy). Self-incompatibility (SI) in plants is, conversely, controlled by a SI locus, named the S-locus. The S-locus has many different alleles and pollen is rejected when it carries the same alleles as the plant being pollinated. Plants self-incompatibility systems can be either gametophytic (GSI) or sporophytic (SSI). In GSI systems, the pollen grain must match either of the alleles present in the diploid maternal tissue to be accepted, making semi-compatibility (mating between half-siblings) possible. In contrast, the pollen grains in the less common SSI must match both of the alleles present in the given female parent to be accepted, making semi-compatibility impossible (Richards 1997).

Inbreeding- and outbreeding depression

Outcrossing in plants is often maintained by inbreeding depression, defined as the reduction of fitness due to selfing or mating between close relatives. The genetic causes of inbreeding depression are usually considered to be due to partial dominance, overdominance, or by combination of both. Partial dominance, which is sufficient to explain most inbreeding depression in higher plants, is the result of expression of deleterious recessive alleles at homozygous loci. The less common overdominance could lead to inbreeding depression, in which heterozygotes have a higher fitness than both types of homozygotes (Charlesworth & Charlesworth 1987; Dudash & Fenster 2000; Keller & Waller 2002). Inbreeding depression is expected to be more pronounced in historically large and outcrossing populations than in mainly selfing populations or in populations that have experienced repeated bottlenecks. The reason for this is that the latter mentioned populations (selfing, repeated bottlenecks) have been purged of their genetic load due to selection (Charlesworth & Charlesworth 1987; Barrett & Charlesworth 1991).

The risk of inbreeding depression is not the only risk that plant populations might be faced with. If plants get pollinated with pollen from distant populations, their offspring might suffer from an outbreeding depression. Two mechanisms are thought to cause outbreeding depression. Firstly crosses with distant pollen might make offspring less adapted to local environments than their parents due to disruption of adaptation to local biotic and abiotic conditions, and secondly, co-adapted gene-complexes might be separated by recombination, also causing outbreeding depression (Lynch 1991; Dudash & Fenster 2000; Hufford & Mazer 2003).

Mating system of plants and their seed dispersal capacities can affect magnitude and timing of inbreeding- and outbreeding depression within plant populations. Inbreeding depression is generally thought to have less impact on selfing species compared to mainly outcrossing species since homozygotes carrying deleterious recessive alleles are exposed to natural selection and may therefore be purged from populations that have gone through generations of inbreeding (Lande & Schemske 1985; Husband & Schemske 1996). Moreover, inbreeding depression often becomes visible in later life-stages in selfers, whereas outcrossing species frequently exhibit inbreeding depression during early stages of their life-cycle. The appearance

of inbreeding depression in early life-history in outcrossing species is caused by recessive lethal mutations in one or a few loci, which are effectively purged from selfing loci (Husband & Schemske 1996). While selfing species are not considered sensitive to inbreeding depression, they are expected to be more sensitive to outbreeding depression than outcrossing species. The dispersal capacity is, moreover, important, since dispersal limited species are thought to be particularly sensitive to outbreeding depression (Dudash & Fenster 2000).

Alpine landscapes and genetic variability

The large number of fragmented microhabitats within the alpine landscape restricts gene flow among plant populations as well as lowering colonization opportunities of new sites (Cain et al. 2000; Theurillat & Guisan 2001). Restricted gene flow together with stronger selection pressures at higher altitudes could make within-population genetic diversity of alpine plants lower and genetic differentiation higher, compared to plants of lower altitudes and less fragmented landscapes. The opposite may, nevertheless, occur (high within-population diversity at higher altitudes) due to the high environmental heterogeneity over small distances and high temporal variation of alpine habitats (Till-Bottraud & Gaudeul 2002).

The population sizes of plants and their type of mating system can, moreover, alter genetic diversity and genetic differentiation. Genetic drift, defined as random changes in allele frequencies, can be enhanced in small populations, which could lead to the extinction of alleles and loss of genetic variability (Barrett & Kohn 1991; Ellstrand & Elam 1993; Lowe et al. 2004). In addition, plants reproductive systems can have a high impact on genetic variability of plant populations. Outcrossing species usually have a high within-population diversity and low population differentiation, whereas selfing species often have low within population diversity and high differentiation among populations (Hamrick & Godt 1996; Booy et al. 2000).

THIS DOCTORAL THESIS

Aims & Objectives

The work presented in this thesis forms part of a larger project "How patchy habitat and isolation affect alpine plant life: genetic diversity, gene flow and mating systems", which includes the PhD studies of Patrick Kuss and the author under the supervision of Professor Jürg Stöcklin.

This doctoral thesis investigates the consequences of the natural fragmentation and patchiness of alpine landscapes on the life of alpine plant populations. The central focus of the thesis is on the mating system, the role of inbreeding and/or outbreeding depression, genetic diversity and geographic structure within and among populations of the rare Alpine monocarpic perennial *Campanula thyrsoides*. The main objectives and research questions addressed are:

- Is *Campanula thyrsoides* self-compatible (SI) and if not, does the SI system break down with flower age? Do inbred *C. thyrsoides* offspring in the common garden suffer from inbreeding depression?
- Do we find a distance related inbreeding depression (poorer reproducive output) or outbreeding depression (increased reproductive output) in field populations of *C. thyrsoides* following crosses of different crossing distances (selfing, 1m, 10m, 100m and among distant populations)?
- How much genetic diversity exists within populations of *C. thyrsoides* and how does it relate to population size and altitude? Has the natural habitat fragmentation let to strong genetic differentiation and restricted gene flow among populations of *C. thyrsoides* resulting in a pronounced geographic structure?

Study species

In order to seek answers to our research questions, we choose to study a yellow bellflower; *Campanula thyrsoides*. The choice was based on the information

that *C. thyrsoides* is a rare plant species, which is only found on calcarious soils within the European Alps and adjacent mountain ranges (Aeschimann et al. 2005). The plants selectiveness for carbonate bearing soils together with the fact that its seeds are not adapted to long-distance dispersal (Tackenberg 2003) are the main reasons for the isolation and small sizes of many of its populations. These population characteristics, therefore, made *C. thyrsoides* a suitable study species. Another important characteristic of *C. thyrsoides*, and one of the main reasons for its inclusion in the study is because it is a monocarpic perennial which flowers once and subsequently dies (Jäger 2000). Monocarpic plants species, which are more commonly found in subtropical and tropical mountain systems (e.g. the giant rosettes of *Puya* spp, *Espeletia* spp., *Echium* spp. etc., Smith & Young 1987; Young & Augspurger 1991) are rare amidst the temperate alpine flora (for the Alps, see Aeschimann et al. 2005). Monocarpy can promote genetic differentiation between populations by reducing the effective population size due to a shorter generation time and lower density of populations (Loveless & Hamrick 1984; Vitalis et al. 2004).

When studying the effects of population isolation and habitat fragmentation on plant reproduction (e.g. mating system and inbreeding depression), it is, moreover, ideal to study a *Campanula* species. Although most *Campanula* species are self-incompatible and allogamous (Nyman 1993), both a break-down in the SI system with flower age (Vogler et al. 1998) and an evolution towards complete self-compatibility (Ægisdóttir & Thórhallsdóttir 2006) have been recorded.

Design

We studied the reproductive ecology and genetic diversity of *Campanula thyrsoides* by firstly setting up pollination experiments in the common garden and in the field and secondly by sampling leaf material in 32 field populations in Switzerland. In the common garden study, we set up a pollination experiment in order to study the breeding system of *C. thyrsoides*, including the consequences of selfing, half-sibling crossings and outcrossing on reproductive output and seedling performance. Moreover, field experiments in four populations were set up in the Swiss Alps in order to study the effect of different crossing distances on reproduction in *C. thyrsoides* and to see if evidence would be found of hidden inbreeding depression or outbreeding depression following large-distance crossings compared to

within-population crossings. In addition, we studied the genetic diversity, gene flow and geographical structure within and among 32 field populations of *C. thyrsoides* in Switzerland, covering both large geographical and altitudinal ranges. The genetic study was conducted using 5 co-dominant microsatellite markers. In addition, we studied the genetic diversity in *C. thyrsoides* and two other alpine plants using random amplified polymorphic DNA (RAPD) marker as well as studing the evolutionary demography of *C. thyrsoides*.

Outline

This thesis is divided into a general introduction (Chapter 1), a summary and a general discussion (Chapter 9) and seven Chapters (no. 2 – 8), which were written for publication in peer-reviewed scientific journals. Outline of Chapter 2-8, publication status and co-authorship is given below.

Chapter 2 **H.H. Ægisdóttir**, D. Jespersen, P. Kuss, J. Stöcklin
No inbreeding depression in an outcrossing Alpine species: the breeding system of *Campanula thyrsoides*
Flora (2007) 202: 218-225

The aim of this study was to study the breeding system, including the consequences of selfing and half-sibling mating in *Campanula thyrsoides*. For this purpose, we set up a pollination experiment in the common garden with plants grown from seeds originating from 14 motherplants (seed families) from the Central Swiss Alps. The experiment included the following treatments: spontaneous selfing, hand-selfing, late selfing (test for break down in self-incompatibility system), half-sibling mating (flowers hand-pollinated with pollen from the same motherplant), outcrossing (flowers hand-pollinated with pollen from another motherplant) and control. We sorted, counted and weighed the seeds and performed germination tests. We, moreover, tested if offspring from outcrossing and half-sibling crossing treatments showed indications of inbreeding depression by comparing seedling survival and the size of outcrossed and sister-crossed offspring.

Chapter 3 **H.H. Ægisdóttir**, P. Kuss, J. Stöcklin

Pollination-distance effect on reproductive within and Between populations of a rare monocarpic perennial plant from the Alps

In preparation

In this chapter, we set up two field experiments over two field seasons in order to study the effect of different crossing distances within a population (selfing, 1m, 10m, 100m) and between populations (distances between populations: 3-113 km) on reproductive output (seed set, seed:ovule ratio, seed germination) in *C. thyrsoides*. We asked firstly if the self-incompatibility system of *C. thyrsoides* is strictly fixed, secondly, if we find a distance related inbreeding depression within *C. thyrsoides* populations, and lastly if we find evidence of hidden inbreeding depression or outbreeding depression in flowers following large-distance crosses compared to within-population crosses.

Chapter 4 **H. H. Ægisdóttir**, B. Koller, P. Kuss, J. Stöcklin

Development and characterization of microsatellite DNA markers for the Alpine plant species *Campanula thyrsoides*

Molecular Ecology Notes (2007) 7 (6): 996-997

This chapter describes the isolation and characterization of eight polymorphic microsatellite markers for *Campanula thyrsoides*. The markers entitled us to study the genetic diversity within and among populations of *C. thyrsoides* with a co-dominant marker (see Chapter 5).

Chapter 5 **H.H. Ægisdóttir**, P. Kuss, J. Stöcklin

High genetic diversity and moderate population differentiation despite natural fragmentation in a rare monocarpic alpine plant

Annals of Botany, submitted

In this chapter, we aimed at an understanding of the effect of natural fragmentation and isolation on genetic diversity, gene flow and geographic structure in *Campanula thyrsoides.* We used 5 microsatellite loci to analyze 736 individuals, originating from 32 populations in the Swiss Alps. We asked how the genetic diversity within populations is related to population size and altitude and if the inbreeding coefficient indicates inbreeding within our studied populations. Furthermore, we asked how much genetic diversity is distributed among populations and used Bayesian approaches to look for geographical structure in our dataset. Lastly, we measured pollen flow directly in the field using fluorescent powder.

Chapter 6 P. Kuss, A.R. Plüss, **H.H. Ægisdóttir**, J. Stöcklin
Spatial differentiation and genetic differentiation in naturally fragmented alpine plant populations
In preparation

This chapter, presents a comparison of genetic diversity and differentiation in three plant species: *Campanula thyrsoides*, *Epilobium fleischeri* and *Geum reptans,* all species found in the Swiss Alps but with different adaptation to long-distance dispersal. All species were studied using the method; Random Amplified Polymorphic DNA (RAPD) in total 400 individuals from 20 study sites in the Swiss Alps for each species. We asked if the fragmentation and isolation of alpine plant populations, has led to an increased population differentition, increasing with distance, and a decreased genetic diversity within populations.

Chapter 7 P. Kuss, M. Rees, **H.H. Ægisdóttir**, S.P. Ellner, J. Stöcklin
Evolutionary demography of the long-lived monocarpic perennial *Campanula thyrsoides* in the Swiss Alps
Journal of Ecology, in revision

In this chapter, we studied how the demography of the long-lived monocarpic perennial *Campanula thyrsoides* influences the size and age at flowering in a temperate alpine mountains system, which is largely devoid of monocarpic plants. We

combined permanent plot and herb chronology data from two populations in the Swiss Alps to parameterize integral projection models (IPMs).

Chapter 8 P. Kuss, **H.H. Ægisdóttir**, J. Stöcklin

The Biological Flora of Central Europe: *Campanula thyrsoides* L.

Perspectives in Plant Ecology, Evolution and Systematics (2007) 9 (1): 37- 51

This chapter reviews all of the existing information on *Campanula thyrsoides*. The information included the taxonomy, morphology, distibution, life cycle, population biology, and genetics of this species as well as its status in the European countries.

Chapter 9 In this last chapter, the main findings in this thesis are discussed, ending with concluding remarks.

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Chapter 2

No inbreeding depression in an outcrossing Alpine species: the breeding system of *Campanula thyrsoides*

Hafdís Hanna Ægisdóttir, Daniela Jespersen, Patrick Kuss

& Jürg Stöcklin

Flora (2007) 202: 218-225

Abstract

Plants that live in fragmented landscapes, where populations are isolated from each other and in which long-distance dispersal is essential for colonization of empty sites, reproduction should be favoured by self-compatibility (Baker's law). Nevertheless, outcrossing mechanisms, such as self-incompatibility and dichogamy, are common in many species and are often maintained by inbreeding depression in the fitness of selfed progeny. Here, we studied the breeding system and the consequences of selfing and sister mating in *Campanula thyrsoides*, a perennial monocarp, which is found in the naturally fragmented landscape of the Alps. An experiment with controlled pollinations was set up in the common garden with plants grown from seeds originating from 14 seed families, collected in the siliceous Central Alps, where this plant is found on isolated carbonate bearing outcrops.

Our results indicate that *C. thyrsoides* has a strong self-incompatibility system (SI) with no or low seed set in selfed flowers compared to outcrossed and sister-crossed flowers. Moreover, the SI system in *C. thyrsoides* did not break down with flower age as in some other *Campanula* species. Surprisingly, there was no significant difference in seed set, seed weight, germination percentage, seedling survival and size between outcrossed and sister-crossed offspring, which indicates no inbreeding depression.

We suggest that the absence of inbreeding depression in this outcrossing species might be a result of frequent bottlenecks during colonization of the isolated habitats in the alpine landscape.

Keywords: Alpine plants; Bottlenecks; Fragmented landscape; Self-incompatibility; Swiss Alps

Introduction

Self-compatibility is frequent among plants, despite the fact that many monoecious or hermaphroditic plant species have mechanisms which promote outcrossing and prevent self-fertilization, i.e. dichogamy, heterostyly or self-incompatibility system (Byers and Waller, 1999; Lande and Schemske, 1985; Richards, 1997). The outcrossing habit of these species is often maintained by inbreeding depression, defined as a reduced fitness of selfed progeny due to the expression of mostly recessive deleterious mutations in homozygotes (partial dominance) or a decrease in heterozygotes that exhibit a fitness advantage over homozygotes (overdominance) (Frankham et al., 2002; Lande and Schemske, 1985). Consequently, historically large and outcrossing populations are expected to have substantial inbreeding depression. In contrast, predominantly selfing populations or populations that have experienced repeated bottlenecks are expected to have lower inbreeding depression due to selection, i.e. the populations have been purged of their genetic load (Barrett and Charlesworth, 1991; Charlesworth and Charlesworth, 1987; Karron, 1989; Ouborg and van Treuren, 1994).

Alpine habitats are characterized by great natural fragmentation and patchiness created by heterogeneous topography and related abiotic factors (Körner, 1999, 2001). As a result, alpine plant populations are often spatially isolated from each other, frequently by long distances. Establishment of new populations in the fragmented and heterogeneous alpine landscape therefore depends on rare but critical long-distance dispersal (Cain et al., 2000; Harper, 1977). Consequently, in isolated populations of species living in a fragmented landscape, self-compatibility should be favoured to ensure sexual reproduction (Baker's law, Baker, 1955, 1967; Jain, 1976).

Most *Campanula* species are self-incompatible (SI) and allogamous (Nyman, 1993; Shetler, 1979), but complete self-compatibility has also been recorded, e.g. in the arctic *C. uniflora* (Ægisdóttir and Thórhallsdóttir, 2006). Self-sterility in plants is controlled by a self-incompatibility locus, named the S-locus that could be comprised of one or several loci. The S-locus has many different alleles and pollen is rejected when it carries the same alleles as the plant being pollinated. This self-incompatibility system can either be gametophytic (GSI) or sporophytic (SSI). In GSI systems, the pollen grain must match either of the alleles present in the diploid maternal tissue to

be accepted, making semi-compatibility (sister mating) possible. In contrast, the pollen grains in the less common SSI must match both of the alleles present in the given female parent to be accepted, making semi-compatibility impossible (Richards, 1997; Silvertown and Charlesworth, 2001). It has been stated that some plants show variation and plasticity in SI as is the case in *Campanula rapunuculoides,* where self-fertility increases with flower age, i.e. delayed selfing (Richardson et al., 1990; Vogler et al., 1998).

In the genus *Campanula,* an interesting mechanism of protandrous flowers has evolved. In the male phase the pollen form a sheath around the hairy style before the stigma becomes receptive. In some *Campanula* species, late in the female phase, the stigmatic lobes bend backwards towards the style picking up pollen that had not been removed by insects. This process facilitates the late self-fertilization of ovules that have not been fertilized by outcrossing (Ægisdóttir and Thórhallsdóttir, 2006; Fægri and van der Pijl, 1979).

Our study species, *Campanula thyrsoides*, is a monocarpic perennial, living at high altitudes in the Alps, frequently in isolated habitats of calcareous soils. Although most *Campanula* species are self-incompatible and allogamous, self-compatible species should be favoured in plants living in patchy alpine habitats (Baker's law), as described above. Moreover, inbreeding depression is an important force for maintaining outcrossing in plants (Frankham et al., 2002; Lande and Schemske, 1985). We therefore expect that inbred *C. thyrsoides* offspring suffer from inbreeding depression. For these reasons, we were interested in the following questions: (1) Is *C. thyrsoides* self-compatible? (2) If it isn't, does the SI (self-incompatibility) system break down with flower age? (3) Do inbred *C. thyrsoides* offspring suffer from inbreeding depression in seed set, seed weight, seed germination and seedling fitness?

Material and methods

Study species

Campanula thyrsoides is a subalpine to alpine monocarpic perennial, found on calcareous soils at about 1300-2800 m asl throughout the European Alps (Lauber and Wagner, 2001). The species is rare but locally abundant with population sizes ranging from less than a hundred to more than 50,000 individuals. Prior to flowering, *C. thyrsoides* forms a basal rosette which overwinters and grows without producing

flowers for about 2-15 years (Kuss et al., in press). In contrast, plants grown in greenhouses occasionally flower in their second year (pers. obs.). In the year of flowering, a 10-40 cm tall inflorences is formed that carries about 50-200 flowers in a compact spike (Kuss et al., in press) (Fig. 1). The flowers are protandrous and mainly pollinated by Hymenoptera, e.g. bumblebees and wasps. The plant dies after setting many small seeds in a multiseeded capsule (average seeds/capsule = 200) (Jäger, 2000; Kuss et al., in press).

Campanula thyrsoides is predominantly found in pastures, extensively used hay-meadows and disturbed areas, such as road sides. In Switzerland, it is common in the northern calcareous Alps, while in the central siliceous Alps, it is only found in isolated carbonate-bearing outcrops (Kuss et al., in press; Lauber and Wagner, 2001).

Fig. 1: Flowering *Campanula thyrsoides* in the Swiss Alps (Photo: Jürg Stöcklin).

Breeding system experiment

In the summer of 2002, we sampled seeds from 30 *C. thyrsoides* plants on the Furkapass, Switzerland (SUI 674850/158825, 2430 m asl). This population is about

25 km away from the nearest *C. thyrsoides* population. The area's main soil type is of siliceous origin, but small lenses of calcareous soil also occur, on which *C. thyrsoides* can be found. We germinated seeds from each of the 30 motherplants and potted 15 seedlings per plant (seed family). Of the total 450 offsprings, only 73 individuals flowered in 2004, of which 41 plants were used in the pollination experiment (3-5 plants per seed family stemming from 12 mother plants). Prior to flowering, we bagged the plants within 50 cm long insect excluders made of green 0.8 mm mosquito nets. The bag was stabilized through two about 110 cm long crosswise attached iron wires in order to avoid direct contact between the bags and flowers.

To explore the breeding system and the consequences of selfing and sister mating in *C. thyrsoides*, we performed controlled pollinations with 4 treatments and 3 replicates by randomly choosing flowers of a single plant, i.e. we applied different treatment on the same flowering individual. The treatments were: a) spontaneous selfing: flowers from within the insect excluders left untreated to test whether seeds are produced in the absence of pollinators, b) hand-selfing: flowers pollinated with their own pollen on the second day of flowering to assess whether insect visitation was required for self-pollination, c) sister mating: flowers handpollinated on the second day of flowering with pollen from plants belonging to the same seed family, and d) outcrossing: flowers handpollinated on the second day of flowering with pollen from plants belonging to another seed family.

Additionally, we left 15 control plants from 14 seed families untreated and unbagged to establish the natural level of seed set. As in the experimental plants, we sampled 3 randomly chosen flowers from each plant. In order to test whether the self-incompatibility system would break down with flower age, we included 30 plants from 10 seed families, bagged them within insect excluders, and hand pollinated them with their own pollen just before the flower withered. We removed the insect excluders as soon as all the flowers, used for the experiment, had withered. In late summer, we collected the seeds, measured plant height, and counted the number of all capsules per plant. We categorized the seeds into a) well developed seeds (round, well filled), b) less developed seeds (flat, not well filled), and c) aborted seeds (very small). After sorting and counting the seeds, we determined their mass by weighing all seeds per capsule, instead of weighing every single seed.

Germination test

We conducted a germination test on 60 well developed seeds for each individual and treatment. We put eight control plants (from 6 seed families) and 27 plants from the outcrossed and sister-crossed treatments (11 seed families; 1-4 plants per family) to trial. We placed twenty seeds on each filter paper in petri dishes and moistened with water. The experiment lasted for 25 days and we estimated germination rates weekly. We kept the seeds wet in an incubator with a 12-h photoperiod and 20°/10°C (day/night) temperature.

Greenhouse experiment with F1 offspring

To find out if the offspring from the outcrossing (assumed inbreeding coefficient $F = 0$) and the sister-crossing treatment ($F = 0.125 – 0.25$; see Wright, 1922) showed indication of inbreeding depression, we set up an experiment to compare the seedling survival and size of outcrossed and sister-crossed offspring.

We germinated seeds in petri dishes and transferred the seedlings into seedling trays with soil in the greenhouse (in both cases 288 seedlings from the same 6 seed families comprising 3 plants per seed family). Fifteen weeks after the transfer, we re-potted the seedlings and randomly arranged them in the Botanical Garden of the University of Basel. We recorded survival rate and plant size (rosette diameter and number of leaves) 15 and 25 weeks after re-potting.

Additionally, we germinated seeds from the control and the selfing treatments and handled the offspring (control: 58 offspring, 5 families, 10-13 per family, and selfing: 30 offspring, 6 families, 1-16 per family) as described above.

Data analysis

We performed statistical analyses using R 1.9.0. Prior to analysis, we log (log+1) transformed all data that did not meet the assumptions of an ANCOVA (Zar, 1999). We tested the treatment effect on mean seed number, seed weight, and seed germination with an ANCOVA model categorizing families and treatments as factors and plant size and number of flowers per plant as covariables.

For the analysis, we included only individuals that met the minimum requirement of 20 seeds within at least one of the three capsules. This was done to

exclude individuals where mistakes in handpollination could not be outruled. We also excluded outliers from the analysis (about 1-3 values per test) that alone changed the tests' result.

We seperately performed the statistical analysis for control vs. outcrossed and control vs. sister-crossed treatments. Only plants from seed families that had both control and treatment plants were included in the analysis, i.e. 26 treatment plants (from 11 families, 1-4 plants per family) and 7 control plants (6 seed families, 1-2 plants per family). Two control plants died. We excluded selfed flowers from the analysis since they did not produce any seeds in most cases (see Fig. 2). To avoid pseudoreplication, we used mean number of seeds from the 3 capsules (replicated in each individual/treatment) in the model.

We tested the treatment effect on seedling survival and size of F1 offspring, in the outcrossed vs. sister-crossed plants with an ANOVA model with families and treatments as factors. When the size of the sister-crossed and the outcrossed F1 offspring was compared to the size of the selfed offspring and the controls, we ran the ANOVA with only treatment as a factor since the number and partition of seed families differed greatly between the treatments.

We calculated the self-compatibility index (SCI) as the number of selfed seeds divided by the sum of outcrossed and sister-crossed seeds, following controlled pollinations.

Results

Seed set

Most of the selfed flowers (spontaneous-, hand- or late selfed) set very few or no seeds with an average of five seed per capsule (sum of all seed categories). This led to a great difference in seed set between the treatments (see Fig. 2).

Seed set was prominent in outcrossed/sister-crossed flowers as well as in the control plants. Here, we found no significant difference in the mean number of seeds between control vs. outcrossed and control vs. sister-crossed flowers respectively (well- developed seeds per capsule: $p = 0.33, 0.07$; less developed seeds per capsule: $p = 0.32, 0.86$; early aborted seeds per capsule: $p = 0.67, 0.25$; total no. of seeds: $p = 0.07, 0.13$).

Additional analyses of the outcrossed vs. sister-crossed treatments revealed no significant difference in the mean values of total no. of seeds per capsule ($p = 0.07$), well developed seeds per capsule ($p = 0.44$) and early aborted seeds per capsule ($p = 0.15$). However, the mean number of less developed seeds per capsule was significantly higher in sister-crossed- compared to outcrossed offspring ($p<0.05$).

We detected no significant difference in number of seeds per capsule among the different seed families for total seeds, well developed and early aborted seeds ($p = 0.15$, 0.19, and 0.74, respectively). However, the number of less developed seeds per capsule differed significantly between seed families ($p<0.05$).

Self-compatibility system

C. thyrsoides is semi-compatible since it set as many seeds in outcrossed and sister-crossed flowers. However, when the number of outcrossed and sister-crossed seeds were compared with selfed seeds, the self-compatibility index (SCI) was very low for both young flowers (spontaneous and hand-selfing) (0.021) and old flowers (late selfing) (0.052). This indicates almost complete self-incompatibility among plants carrying the same alleles.

Seed weight

There was no significant difference in seed weight (mg) per capsule between controls vs. outcrossed and controls vs. sister-crossed seeds ($p = 0.36$, 0.86, respectively). Neither could we detect a significant difference between seed families (control vs. outcrossing, $p = 0.36$; control vs. sister crossing, $p = 0.72$). The only difference that we detected was a higher seed weight in sister-crossed seeds compared to outcrossed seeds ($p<0.05$).

Seed germination

The seed germination was very high for both control, outcrossing and sister-crossing treatments. We recorded a 78.8 % germination success in seeds from the control treatment, 88.7% from outcrossing, and 82.7% from sister-crossing (Table 1). Germination percentage for seeds from control plants did not differ significantly from germination percentage observed in seeds from outcrossed and sister-crossed treatments ($p = 0.60$, 0.97, respectively). Slightly fewer seeds from the sister-crossing

treatment germinated compared to seeds from the outcrossing treatment but the difference was only marginally significant (p = 0.07). There was no difference in the percentage of seed germination among seed families (p = 0.91).

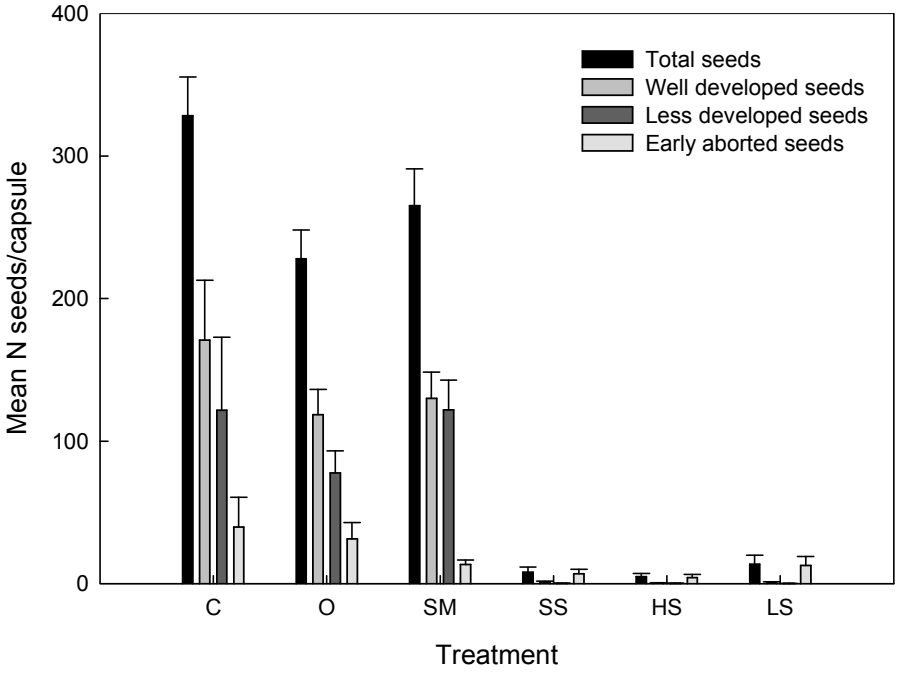

Fig. 2. Number of a) total, b) well developed, c) less developed, and d) early aborted seeds per capsule in control (C), outcrossed (between seed families) (O), sister mated (inbred within seed families) (SM), spontaneously selfed (SS), hand selfed (HS), and late selfed (LS) *Campanula thyrsoides* plants from a pollination study in the common garden. Value represents mean ± standard error (SE). For N see Table 1.

Survival and size of seedlings

Fifteen weeks after the seedlings were transferred, many had died (mortality = 69 %), but the mortality percentage did not differ significantly between the outcrossing and the sister crossing treatments (p = 0.17). Additionally, there was no significant difference in the diameter of rosette (after 15 weeks: p = 0.46, after 25 weeks: p = 0.29) and number of leaves per rosette (after 25 weeks: p = 0.66) between the outcrossed and the sister-crossed plants (Table 2).

Moreover, we detected no significant difference in the rosette diameter (p = 0.14), and number of leaves per rosette (p = 0.35) between the offspring of control, selfed, sister-crossed and outcrossed plants 25 weeks after the seedlings transfer,

although the rosette diameter was significantly larger in selfed offspring after 15 weeks ($p< 0.05$).

Discussion

Breeding system and seed set

C. thyrsoides had the same floral development as most other *Campanula* species being strongly protandrous with pollen deposited by the anthers directly onto the style before bud opening. Later the anthers withered and the stigma became receptive. This kind of temporal separation of male and female maturation, dichogamy, is usually regarded as an outbreeding mechanism (Bhardwaj and Eckert, 2001; Silvertown and Charlesworth, 2001). In our case this was truly so, since the results show us that *C. thyrsoides* is allogamous and self–incompatible like has been recorded to be the most common reproductive mode of the genus (Nyman, 1993; Shetler, 1979). Moreover, the SI system of *C. thyrsoides* proved to be of gametophytic origin since the flowers pollinated with pollen from plants from the same seed family (sister mating) produced as many seeds as outcrossed flowers (called semi-compatibility), but the same has been documented for other species in the Campanulaceae (Richards, 1997; Steinbachs and Holsinger, 2002; Stephenson et al., 1992).

Table 1. Mean weight (mg) of seeds per capsule (± SE) and mean germination percentage (± SE) in *Campanula thyrsoides* after different pollination treatments

Treatment	Weight (mg)	Seed germination (%)	N
Control	16.17 (± 2.42)	78.80 (± 3.93)	7
Outcrossing	13.71 (± 1.59)	88.65 (± 2.85)	26
Sister crossing	15.90 (± 1.84)	82.73 (± 3.85)	26
Hand-selfing	0.09 (± 0.04)		26
Spontaneous selfing	0.14 (± 0.07)		26
Late selfing	0.17 (± 0.08)		26

N = number of plants per treatment.

Table 2. Mean rosette diameter (mm) (± SE) and number of leaves per rosette (± SE), 15 and 25 weeks after 2nd generation *Campanula thyrsoides* seedlings from four different pollination treatments had been re-potted

	Rosette diameter 15 weeks	Rosette diameter 25 weeks	*N* leaves / rosette 25 weeks	N
Control	63 (± 3.17)	176 (± 4,62)	55 (± 1.82)	58
Outcrossing	58 (± 6.61)	191 (± 4.22)	52 (± 2.06)	68
Sister crossing	55 (± 5.42)	181 (± 4.60)	50 (± 1.64)	79
Selfing	65 (± 4.4)	186 (± 7.55)	53 (± 2.37)	30

N = number of seedlings per treatment.

The very low self compatibility index (0.021 for spontaneous and hand-selfing) indicates that *C. thyrsoides* has a very strong SI system. Plants with a self-compatibility index (SCI) of less than 0.15 are defined as strong SI plants while plants with a SCI of more than 0.40 are regarded as weak SI plants (Stephenson et al., 2000). Besides, there was no indication of a break down in the SI system with flower age as has been the case e.g. in *Campanula rapunculoides* (Vogler et al., 1998) since the SCI of the late selfing plants was only slightly higher (0.052) than in the spontaneous and hand-selfing (early selfing) plants.

The strong SI system and the allogamous habit of *C. thyrsoides* should not be very surprising since most *Campanula* species are self-incompatible and allogamous (Shetler, 1979; Nyman, 1993). Since strict self-incompatibility is rare among alpine and arctic plants (Brochmann and Steen, 1999; Grundt et al. 2005; exceptions: e.g. Kelso, 1987; Molau, 1993), a weaker SI system in *C. thyrsoides* is logically expected. For example, *C. uniflora* populations in Greenland and Iceland, occurring under very similar arctic conditions, showed self-compatibility and even preanthesis cleistogamy (Ægisdóttir and Thórhallsdóttir, 2006).

As was previously mentioned, *C. thyrsoides* often lives in small and isolated populations in the fragmented Alpine landscape where self-compatibility should be favoured (Baker, 1955, 1967). Moreover, the species is monocarpic, which also might enhance self-compatibility (Barrett et al., 1996). Nevertheless, this does not seem to favour self-compatibility in *C. thyrsoides*.

Inbreeding depression

Surprisingly, we found no indication of inbreeding depression in *C. thyrsoides* in this study as there was no significant difference in seed set, seed weight, and germination percentage between outcrossed and sister-crossed flowers. Equally, no difference was detected in the survival and size of outcrossed (assumed inbreeding coefficient ($F = 0$)) and sister-crossed offspring ($F = 0.125 – 0.25$). Why did we not detect any inbreeding depression in this outbreeding species? Living in the fragmented landscape of the Alps and consequently being spatially isolated from other populations could have caused frequent bottlenecks during colonization of isolated habitats. Since plant populations that have experienced repeated bottlenecks or pollinator failures are likely to exhibit reduced levels of inbreeding depression due to a reduction in genetic load, this could explain the low inbreeding depression in this outbreeding species. However, repeated bottlenecks might also select for reproductive assurance and thus lead to a break-down of the SI systems (Glémin et al., 2001; Karron, 1989; Lande and Schemske, 1985), but this was not observed in the studied population.

Moreover, Frankham et al. (2002 and references therein) argue that the degree of inbreeding depression also depends upon the amount of inbreeding. In this context, the ideal situtation to compare fitness values between plants is given for individuals with highly contrasting inbreeding coefficients, i.e. $F = 0.5$ for complete selfing and $F = 0$ for complete outcrossing. To recall, *C. thyrsoides* displayed maximum F values in a range of 0.125 – 0.25 and a decrease in fitness measures might have remained undetected due to the small contrast with completely outbred individuals. Inbreeding depression is also sometimes first detected in later stages of the life cycle, such as seedling biomass of the reproduction of second generation progeny (Karron, 1989), which we were not able to follow in this study. It is also possible that the control plants suffered from inbreeding depression because of fixed deleterious alleles. Moreover, since relatively few plants flowered in summer 2003, we can not completely exclude the possibility that those plants were more vigorous and less inbred than the plants which flowered later.

Conclusion

Like most other *Campanula* species, *C. thyrsoides* appeared to be both allogamous and self-incompatible. Since *C. thyrsoides* is a successful outcrosser, we expected to detect some negative inbreeding effects in inbred offspring (sister mating), which was not the case in our study. We conclude that the absence of inbreeding depression in this outcrossing Alpine species might be a result of frequent bottlenecks during colonization of isolated habitats in the fragmented Alpine landscape.

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Chapter 3

Pollination-distance effect on reproduction within and between populations of a rare monocarpic perennial plant from the Alps

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Abstract

Inbreeding and outbreeding depression in plants, which often depend on mating history and dispersal ability, may be crucial for population survival. Outcrossing plant species frequently show inbreeding depression in selfed progeny during early life-stages, and species with limited dispersal capacities are considered to be particulary prone to outbreeding depression.

We studied the effect of different crossing distances (self, 1, 10, 100 m and among distant populations) on the reproductive output in field populations of the rare Alpine *Campanula thyrsoides*, an outcrossing monocarpic plant species with limited seed dispersal capacity. Our main objective was to assess distance related inbreeding depression within *C. thyrsoides* populations, and elucidation of hidden inbreeding depression or outbreeding depression following large-distance among population crosses compared to within-population crosses.

Plants pollinated within populations by pollen donors < 1 m away set significantly fewer seeds than more distant individuals (10 m). This indicates an inbreeding depression in crossings between close relatives. There was, moreover, lower seed set in the 100 m distance separation compared to the 10 m distance, indicating an optimal outcrossing distance of about 10 m. Crossings among populations did neither indicate a hidden inbreeding depression nor an outbreeding depression.

We conclude that for the outcrossing *C. thyrsoides* that occurs in isolated habitats in the alpine landscape, inbreeding depression could be disadvantageous if populations are very small. This may be a reason why only sparsely distributed *C. thyrsoides* populations occur in the Alps.

Keywords

Campanula thyrsoides, inbreeding depression, seed germination, seed set, Swiss Alps

Introduction

Ever since Darwin (1876) published his thoughts and descriptions on the effects of cross and self fertilization in plants, inbreeding depression and its consequences on plants fitness has interested biologists (e.g. Cooper and Brink, 1940; Price and Waser, 1979; Levin, 1984; Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Waser and Price, 1994; Hedrick and Kalinowski, 2000; Becker et al., 2006). The reduction of fitness due to selfing or mating between close relatives (inbreeding depression), is generally thought to be caused by either of two mechanisms that may occur separately or in combination: partial dominance or overdominance. In partial dominance inbreeding depression is the result of expression of deleterious recessive alleles at homozygous loci, whereas overdominance could lead to inbreeding depression in which heterozygotes have a higher fitness than both types of homozygotes (Charlesworth and Charlesworth, 1987; Byers and Waller, 1999; Dudash and Fenster, 2000; Keller and Waller, 2002). Although some evidence exists to support both of the mentioned mechanisms, partially recessive deleterious mutations suffice to explain most inbreeding depression in higher plants (Charlesworth and Charlesworth, 1999; Keller and Waller, 2002).

Mating that occurs among plants separated by large distances, either within or between populations, may have either positive or negative consequences on offspring fitness. One positive effect, which has been recorded in several studies, is an increased vigor of offspring (heterosis) relative to the parental fitness (Oostermeijer et al., 1995; Byers, 1998; Fenster and Galloway, 2000). This increased hybrid vigor may be due to masking of deleterious recessive alleles, which were fixed in the parent population but become harmless in the hybrid population as a result of the heterozygote form. Alternatively, the increase in hybrid vigor could be due to an overall fitness advantage of heterozygotes (overdominance) (Lynch, 1991; Keller and Waller, 2002; Hufford and Mazer, 2003).

The negative effect of large-distance crosses (usually referred to as outbreeding depression) has been recorded over the last century or more (e.g. Müller, 1883; Kruckeberg, 1957; Price and Waser, 1979; Waser and Price, 1994; Fischer and Matthies, 1997; Becker et al., 2006). Outbreeding depression appears to be caused by at least two mechanisms. Firstly, a separation of co-adapted gene-complexes by recombination might occur, resulting in reduced fitness of offsprings. Secondly, large-

distance crosses may disrupt adaptation to local biotic and abiotic conditions, making offspring less adapted to local environments than their parents. Moreover, outbreeding depression is thought to be particularly prevalent in plants that are dispersal limited and in which genetic differentiation between plant populations is high (Price and Waser, 1979; Lynch, 1991; Dudash and Fenster, 2000; Fenster and Galloway, 2000; Hufford and Mazer, 2003)

The magnitude and timing of in- and outbreeding depression depends partly on mating history and dispersal ability of the species and populations in question. Selfing species are generally considered to be less sensitive to inbreeding depression than outbreeding species since homozygotes carrying deleterious recessive alleles are exposed to natural selection and may therefore be purged from populations that have gone through generations of inbreeding (Lande and Schemske, 1985; Husband and Schemske, 1996; Montavlo et al., 1997). In selfers, inbreeding depression often appears in later life-stages, whereas outcrossing species frequently exhibit inbreeding depression during early stages of their life-cycle. This early life-history expression of inbreeding depression is caused by recessive lethal mutations in one or a few loci, which are effectively purged from selfing loci (Husband and Schemske, 1996). In contrast to inbreeding depression, outbreeding depression is expected to be more pronounced in highly selfing species and poorly dispersed species, than in outcrossing species and those which disperse easily between populations (Dudash and Fenster, 2000).

In natural populations of plant species with limited pollen and seed dispersal, genetic structure is produced, which causes plants to be more related to their nearest neighbor than to more distant plants and causes kinship between individual plants to decrease with increasing distance between mates (Turner et al., 1982; Sokal and Wartenberg, 1983; Barbujani, 1987). Consequently, reduced fertility (putative inbreeding depression) is often observed in crosses between near-neighbor plants, compared to crosses among more distanced plants (e.g. Price and Waser, 1979; Levin, 1984; Sobrevila, 1988; Souto et al., 2002).

In this study, we performed two separate experiments to examine whether pollination distances affect reproductive output within and between populations of the alpine *Campanula thyrsoides*, one of few representatives of rosette-forming monocarpic perennials in the European Alps (Aeschimann et al., 2005). We choose to study *C. thyrsoides* due to its monocarpic nature and because of its rareness within the alpine landscape where it is mostly found in small and isolated populations on carbonate

bearing soils (Kuss et al., in press). In an accompanying common garden study, we found that *C. thyrsoides* is mostly outcrossing with a gametophytic self-incompatible system, i.e. it is mostly unable to self-cross but can mate with its half-siblings (Ægisdóttir et al., 2007). Our former studies, moreover, indicated that seed dispersal is particularly limited in *C. thyrsoides* and mostly occurs close to the mother plant (Kuss et al., in press; Ægisdóttir et al., submitted).

We performed controlled pollinations within four populations in Switzerland, testing both for potential selfing and the effects of crossing distances (1 m, 10 m, 100 m) on seed set. Furthermore, we studied the effect of between-population crosses on seed set and seed germination within two populations by crossings their plants with pollen from several populations of different proximity (3-113 km away). We addressed the following questions: a) Is the self-incompatibility system of *C. thyrsoides* strictly fixed? b) Do we find a distance related inbreeding depression within *C. thyrsoides* populations, indicated by poorer reproductive output in flowers from short distance crossings (1 m) compared to crossings of larger distances (10 m, 100 m)? c) Do we find evidence of hidden inbreeding depression (increased reproductive output) or outbreeding depression (decreased reproductive output) in flowers following large-distance crossings (3-113 km) compared to within-population crossings?

Materials and methods

The plant species and study sites

Campanula thyrsoides is a subalpine to alpine species, found on calcareous soils at 1000-2800 meters above sea level (m asl) throughout the European Alps and adjacent mountain ranges (Aeschimann et al., 2005; Kuss et al., in press). In spite of its wide ecological amplitude with regard to habitats (pastures, hay-meadows and disturbed areas, such as along road shoulder), it is rare throughout it native range (Kuss et al., in press). In Switzerland, the species is more common in the northern calcareous Alps, compared to the central siliceous Alps, where it is only found in isolated carbonate-bearing outcrops (Lauber and Wagner, 2001; Kuss et al., in press).

Campanula thyrsoides is a diploid (2n=34) rosette-forming monocarpic plant species which flowers once and then dies. In the year of flowering (mean time of flowering = 7.5 years, range = 3-16 years), a 10-40 cm tall inflorescence is formed that

carries about 50-200 flowers in a compact spike. The plant dies after setting many small seeds in a multiseeded capsule (average well developed seeds per capsule = 147) (Kuss et al., in press). *C. thyrsoides* is an outcrossing species, which has protandrous flowers and a gametophytic self-incompatible system, i.e. it is mostly unable to self but can mate with its half-siblings (Ægisdóttir et al., 2007).

Our accompanying studies on the genetic diversity, gene flow and geographical structure in *C. thyrsoides* in Switzerland showed that the species shows a moderate to high within-population genetic diversity and moderate genetic differentiation among populations (Ægisdóttir et al., submitted; Kuss, 2006). The flowers were mainly pollinated by bumblebees, and direct measures of pollen flow in the field showed that most pollinators flew short distances between flowers (< 2 m), although occasional flights were longer (up to 39 m) (Ægisdóttir et al., submitted). It has also been observed that *C. thyrsoides* has a highly limited seed dispersal capacity. Seed dispersal spectra, obtained from simulations using the software Pappus (Tackenberg, 2003; Tackenberg and Stöcklin, in press), showed that most seeds are dispersed very close to the mother plant (Kuss et al., in press).

Field work was carried out during summer 2004 and 2005 in four populations in Switzerland; two populations from the calcareous Alps and the Jura mountains and two populations located in isolated areas of calcareous soil within the siliceous Alps. The populations were: a) Col du Marchairux (coordinates according to the Swiss topographical maps; SUI: 508' 900/156' 400): The population is located at a rather low altitude (1440 m asl) in the calcareous Jura Mountains in the Southwestern part of Switzerland, b) Lac du Moiry (SUI: 609' 932/109' 638): The population is located on a band of calcareous bedrock within the siliceous Southern Swiss Alps at around 2266 m asl. The population is mostly found at high densities along a hiking trail, c) Furka Pass (674' 850/158' 825): The population is very large, located mostly in high densities in disturbed areas alongside the pass-road. The population is located in the siliceous Alps, in an island of calcareous bedrock, at about 2430 m asl, d) Parsennmeder (784' 030/191' 473). The populations are found in a meadow in the Eastern part of the Swiss Alps, at about 1990 m asl.

Populations were selected in order to include both isolated (on calcareous bedrock within the siliceous Alps) and less isolated (within the calcareous Alps) populations. Since the experimental design required a minimum number of flowering *C. thyrsoides* individuals, only populations of moderate and large sizes could be included.

Crossing design

Crossings within populations

We performed controlled pollinations within the four populations during the summer of 2004. In each population, we used 20 randomly selected plants (10 treatment and 10 control plants). Each plant was marked with small red-flagged wooden stakes. In each control plant, we randomly selected and marked 3 flowers. Thereafter, the control plants were left untreated and unbagged to allow for the natural level of seed set. We bagged all treatment plants already at budding stage inside insect excluders made of nylon curtain material to prevent visits from pollinators. On each treatment plant, we performed 5 different treatments (3 randomly selected flowers per treatment) and marked the flowers with colored pins below the flower to be able to distinguish between different treatments. The treatments were: i) Spontaneous Selfing (SS): the purpose was to establish whether seeds are produced in the absence of pollinators, ii) Hand-Selfing (HS): flowers were hand-pollinated using their own pollen by brushing pollen on the stigma when it was receptive for pollen. This treatment was carried out to assess whether insect visitation was required for self-pollination, iii) Outcrossing (1 m): flowers were hand-pollinated with pollen from a pollen donor located ≤ 1 m from the treatment plant (nearest neighbor), iv) Outcrossing (10 m): flowers were hand-pollinated with pollen from a pollen donor located about 10 m from the treatment plant, v) Outcrossing (100 m): flowers were hand-pollinated with pollen from a pollen donor located ≥ 100 m from the treatment plant. For each crossing, we randomly selected a plant to serve as pollen donor.

Crossings between populations

During summer 2005, we set up a crossing between-population experiment in order to test for a hidden inbreeding depression or for outbreeding depression within populations, i.e. to look for increased or decreased seed set and/or germination in plants pollinated with pollen from distant populations compared to plants pollinated with pollen from within their own population.

Two of the four populations used for the pollination experiments in summer 2004 were used for this study: Col du Marchairux (West Switzerland) and Parsennmeder in the eastern Swiss Alps. In both populations, ten randomly chosen plants were marked with wooden stalks, and 3 flowers per treatment randomly selected

and color-marked. Since it was not possible to standardize the crossing distances for the two study sites, we considered the two sites as separate experiments.

In Col du Marchairux the flowers were crossed with: a) Pollen from flowers within the same populations (control), b) Pollen from a population close by (Short distance: 3 km away), and c) Pollen from a population more distant (Middle distance: 13 km away). In Parsennmeder, the flowers were crossed with: a) Pollen from flowers within the same populations (control), b) Pollen from a population close by (Short distance: 6 km away), c) Pollen from a population more distant (Middle distance: 23 km away), and d) Pollen from a population far away (Long distance: 113 km away). For each crossing, we randomly selected a plant to serve as pollen donor.

Offspring performance

As soon as all flowers on the experimental plants had withered, we removed the insect excluders. In late summer 2004 and 2005, we collected the seed capsules just before capsule opening. We categorized the seeds as 'well developed seeds' (round, well filled and dark seeds) and 'less developed seeds' (flat, incompletely filled seeds) and subsequently counted them. In order to be able to calculate the seed:ovule ratio (SO ratio), we counted all aborted seeds (very small seeds) and unfertilized ovules. In addition, we calculated the autodeposition efficiency as the mean SO ratio in spontaneously selfed plants relative to the mean SO ratio of control plants (Molau, 1993). On each control and experimental plant, we measured co-variables to be able to control for plant size in statistical analyses. We further recorded the height of flowering stalk and inflorences (mm) and the total number of capsules per plant.

Seeds from both pollination experiments were tested for successful germination. Unfortunately, very few seeds from the 2004 pollination experiment (within-population crosses) germinated, most likely because of too early harvest, i.e. the seeds were not completely ripe. However, seeds from the 2005 pollination experiment (between-population crosses) germinated, allowing us to conduct a germination test on 60 well-developed seeds for each individual and treatment. We placed twenty seeds (3 replicates for each individual and treatment = 60 seeds) on each filter paper in petri dishes and moistened them with water. The experiment lasted for 28 days and we estimated germination rates weekly. Seeds were kept wet in an incubator with a 12-hour photoperiod and 20°/10°C (day/night) temperature.

Statistical analysis

Prior to analysis, we log (log+1) transformed all data that did not meet the assumptions of an ANCOVA test (Zar, 1999). For the crosses within-population experiment, we tested the treatment effect on the mean number of well- and less developed seeds per capsule as well as the number of all developed seeds (sum of well- and less developed seeds) and SO ratio. These were tested using a mixed model analysis of covariance (ANCOVA) with the following order of covariate terms: plant height, number of capsules per plant, population, treatment, population × treatment interaction. Treatment-effects were tested against the error stratum population × treatment, while population × treatment was tested against the residuals. Since the interaction; treatment × population was never significant, we concluded that the treatment effect of the pollinations were the same in all four study populations. We therefore present results of pooled data from all four populations. Statistical analysis were performed on the following a priori comparisons: 1) Outcrossing 1 m vs. outcrossing 10 m vs. outcrossing 100 m, 2) 1 m vs. 10 m, 3) 1 m vs. 100 m, 4) 10 m vs. 100 m.

For the crossing between-population experiment, we tested the treatment effect on the mean number of well- and less developed seeds per capsule as well as the number of all developed seeds (sum of well- and less developed seeds), SO ratio and seed germination. We performed two separate statistical analyses for the two populations (Col du Marchairux and Parsennmeder) since the populations were considered as two separate experiments. For both populations, we tested the treatment effect using a mixed model analysis of covariance (ANCOVA) with the following order of covariate terms: plant height, number of capsules per plant, treatment. Treatment-effects were tested against the residuals.

To avoid pseudoreplication, we used mean number of seeds from the 3 capsules (replicated in each individual/treatment) in the model. All statistical analyses were performed using R 2.0.0 (R Development Core Team 2004).

Results

Selfing

We found that selfing treatments (spontaneously selfing and hand-selfing) resulted in much lower seed set compared to the outcrossing treatments, leading to a

large difference in seed set among treatments (Fig 1). Nevertheless, there was some seed set in the selfing treatment, with an average of 16.2 seeds per capsule (the sum of all developed seeds) and 56 % of the capsules setting ≥ 1 seed in the spontaneous selfing (SS) treatment. In the hand-selfing treatment, there were ten developed seeds per capsule and 41 % of capsules setting ≥ 1 seed. In comparison, an average seed set in the control flowers was 191 seeds per capsule. Moreover, the autodeposition efficiency of spontaneously selfed plants was 0.19.

Distance crossings within populations

The outcrossing treatments had a significant effect on the number of well- and all developed seeds ($p < 0.05$, Table. 1, Fig. 1). Interestingly, seed set was lower in the 1 m treatment compared to the 10 m treatment for well- and all developed seeds ($p < 0.05$). Additionally, the 100 m treatment set fewer seeds (well- and all developed seeds) and had lower SO ratio than the 10 m treatment ($p < 0.05$, Table 1, Fig. 1).

Seed set in the control plants was generally not lower compared to the outcrossing treatments (see Fig. 1), indicating that the control plants were not pollen limited.

Distance crossings among populations

At both Col du Marchairux and Parsennmeder there were no significant differences in seed set (all seed categories), SO ratio, and seed germination between the control plants and the treatment plants (Table 2, Fig. 2).

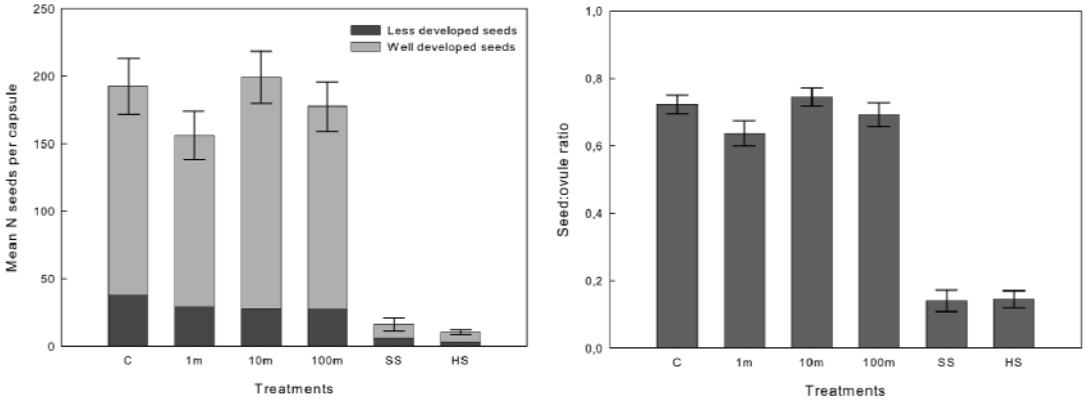

Figure 1: Crossings within populations: a) Number of less- and well developed seeds per capsule (left) and b) seed:ovule ratio (right) in the following treatments: control (C), outcrossing <1m away (1m), outcrossing 10m away (10m) and outcrossing >100m away (100m), spontaneous selfing (SS) and hand-selfing (HS) on *Campanula thyrsoides* plants in the Swiss Alps. Values represent mean ± standard error (SE).

Size differences between populations

The four study populations did not differ in their responses to the crossing distances within populations, as the interaction between treatments and populations was never significant. However, the populations differed significantly in the number of well developed seeds ($F = 16.1$, $p < 0.001$) and all developed seeds ($F = 26.17$, $p < 0.001$) per capsule in the control plants. This difference was due to higher number of seeds per capsule in the plants at the Furka Pass study populations compared to the other study sites (Table 3).

The Furka plants had, on average, the highest inflorescence (mm) and the highest number of flowers per plant (Table 3). The size parameters of the plants were significantly correlated with the number of well developed seeds (height of inflorescence, $r = 0.35$, $p < 0.05$; number of flowers per plant, $r = 0.4$, $p < 0.05$).

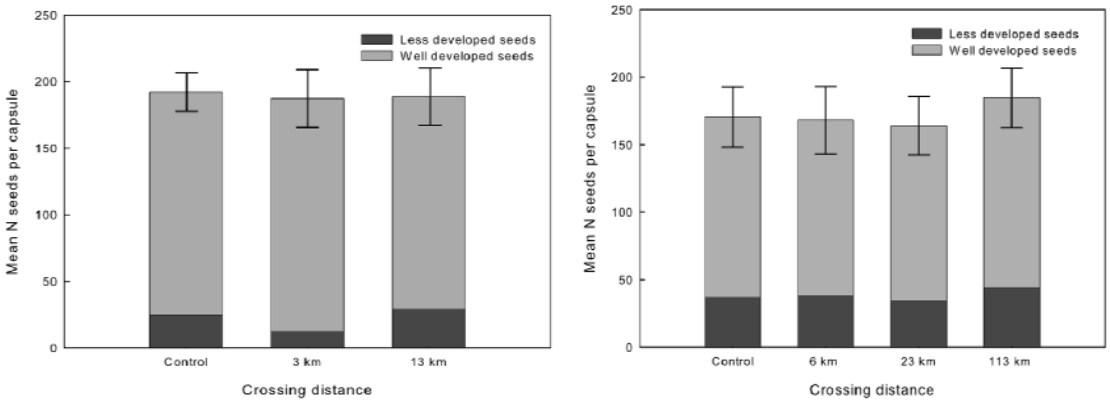

Figure 2: Crossing between populations: Number of well- and less developed seeds per capsule in the following treatments for a) Col du Marchairux (left): control, short distance (3 km away) and middle distance (13 km away), b) Parsennmeder (right): control, short distance (6 km away), middle distance (23 km away) and long distance (113 km away) in *Campanula thyrsoides* Values represent mean ± standard error (SE).

Discussion

In this study, we investigated the effect of different distances of intra- and inter-population crosses on reproduction in *C. thyrsoides* in the field. We especially looked for indication of inbreeding and/or outbreeding depression within the plants' populations. Pollination distance affected seed set within populations, indicating an inbreeding depression in short-distance crossings and an optimal outcrossing distance of about 10 m. However, crossings between populations did neither result in an increased nor a decreased reproduction, indicating an absence of any hidden inbreeding depression as well as outbreeding depression within our studied populations.

Selfing (both spontaneously and hand-selfing) resulted in much lower seed set compared to the control and outcrossed plants (Fig. 1). Nevertheless, seed set in selfed capsules was still found to be greater than in a common garden experiment (Ægisdóttir et al., 2007). Given that the autodeposition efficiency of spontaneously selfed flowers was 0.19 and that 56 % of spontaneously selfed flowers set 1 or more seed per capsule, it is reasonable to conclude that the self-incompatibility system in *C. thyrsoides* is not strictly fixed. Although most *Campanula* species are self-incompatible (SI) and outcrossing, some SI species (including *Campanula rapunculoides*) show plasticity or

Table 1: Crosses within populations of *Campanula thyrsoides*: *F* values and significance levels from ANCOVA with well-developed, less-developed and all developed seeds (sum of well- and less developed seeds) per capsule, as well as SO ratio. Sources include the following treatments and interaction between population and treatment: a) three outcrossing treatments (1m vs. 10 m vs. 100 m away), b) 1m vs. 10m, c) 1m vs. 100m, and d) 10m vs. 100m. ns = not significant, level of significance: * ($p < 0.05$).

Comparison	Source	Well developed seeds (a)	Less developed seeds (b)	All developed seeds (a+b)	SO ratio
1m vs. 10m vs. 100m	Treatment	9.50 *	1.10 ^{ns}	10.45 *	3.53 ^{ns}
	Population × treatment	0.16 ^{ns}	0.14 ^{ns}	0.12 ^{ns}	0.42 ^{ns}
1m vs. 10m	Treatment	12.09 *	1.30 ^{ns}	12.10 *	4.60 ^{ns}
	Population × treatment	0.40 ^{ns}	0.23 ^{ns}	0.32 ^{ns}	1.13 ^{ns}
1m vs. 100m	Treatment	4.29 ^{ns}	0.27 ^{ns}	5.40 ^{ns}	1.58 ^{ns}
	Population × treatment	0.30 ^{ns}	0.27 ^{ns}	0.20 ^{ns}	0.63 ^{ns}
10m vs. 100m	Treatment	28.61 *	3.95 ^{ns}	27.08 *	11.11 *
	Population × treatment	0.02 ^{ns}	0.08 ^{ns}	0.02 ^{ns}	0.07 ^{ns}

Table 2: Crossings between populations of *Campanula thyrsoides*: Seed:ovule ratio (SO ratio ±SE) and percent seed germination (±SE) in the two following populations: a) Col du Marchairux: control and two treatments (crossings: 3 km and 13 km) and b) Parsennmeder: control and three treatments (crossings: 6 km, 23 km and 113 km).

Site	Treatment	SO ratio	Seed germination (%)
Col du Marchairux	Control	0.81 (± 0.03)	35.00 (± 8.33)
	3 km	0.73 (± 0.05)	24.26 (± 8.88)
	13 km	0.84 (± 0.03)	24.63 (± 7.80)
Parsennmeder	Control	0.66 (± 0.07)	10.74 (± 3.07)
	6 km	0.69 (± 0.06)	7.04 (± 1.10)
	23 km	0.71 (± 0.05)	13.89 (± 4.57)
	113 km	0.73 (± 0.05)	20.19 (± 6.50)

Table 3: Mean (±SE) height of inflorescences (mm), number of flowers per plant, mean number of well developed seeds per capsule and seed:ovule ratio (SO ratio) at four study sites (Furka Pass, Parsennmeder, Lac du Moiry and Col du Marchairux) of *Campanula thyrsoides* in the Swiss Alps.

Study sites	Height inflorescence	# flowers/plant	# seeds/capsule	SO ratio
Furka Pass	178 (± 17.5)	84 (± 8.0)	288 (± 37.69)	0.91 (± 0.02)
Parsennmeder	121 (± 12.8)	61 (± 4.1)	135 (± 17.74)	0.70 (± 0.05)
Lac du Moiry	116 (± 13.5)	44 (± 4.1)	111 (± 21.03)	0.76 (± 0.03)
Col du Marchairux	167 (± 18.3)	62 (± 5.5)	80 (± 13.20)	0.57 (± 0.04)

variation in the strength of SI (Richardson et al., 1990; Vogler et al., 1998; Goodwillie, 1999). Our results suggest to some extent that plasticity or variation in the SI system is favorable for the monocarpic *C. thyrsoides*. It allows for at least minimal seed set in the absence of pollinators in unfavorable years and buffers the effect of spatially isolation of population (Baker, 1955, 1967; Ghazoul, 2005).

Interestingly, our results show that the 10 m crossing distance positively affected reproductive output in *C. thyrsoides*. Since most pollen and seeds are dispersed in close vicinity of the mother plant (Ægisdóttir et al., submitted; Kuss et al., in press) we expect

neighboring plants to be more genetically related than plants further away (Turner et al., 1982; Sokal and Wartenberg, 1983; Barbujani, 1987). This suggests that our results indicate a certain degree of inbreeding depression in the 1 m treatment plants, most likely due to mating between closely related individuals (e.g between half-siblings). Similarly, inbreeding depression after pollination with near neighbors has been recorded for a number of plant species (Price and Waser, 1979; Levin, 1984; Dudash, 1990; Waser and Price, 1994). Moreover, some selfing and mating between closely related individuals is in accordance with our recorded significant heterozygote deficiency in eleven out of 32 studied populations of *C. thyrsoides* in the Swiss Alps (Ægisdóttir et al., submitted).

The significantly lower seed set and SO ratio following the 100 m compared to the 10 m crosses (Table 1, Fig. 1) indicates an optimal outcrossing distance at about 10 m distance for *C. thyrsoides*. Many studies on plants point out that crosses over intermediate distance (usually 3-10 m) result in highest fitness compared to both shorter (selfing and 1 m) and longer (100 m and 1000 m) distances, e.g. in *Delphinium nelsonii* (Price and Waser, 1979; Waser and Price, 1991, 1994), *Ipomopsis aggretata* (Waser and Price, 1989) and *Gentianella germanica* (Fischer and Matthies, 1997). This superiority of intermediate crossing distances led Price and Waser (1979) to state that an "optimal outcrossing distance" exists within populations, which is defined as the distance where seed set reduction due to inbreeding and outcrossing is not present. This optimal outcrossing distance should depend on the spatial scale of differentiation within and between populations, and on the average distance of gene flow (Price and Waser, 1979).

For *C. thyrsoides*, an optimal crossing distance of about 10 m away from the mother plant seems to fit with our pollen-flow test (using fluorescent powder) since the pollen-flow result showed that pollinators contributed greatly to pollen flow in *C. thyrsoides* and that most of pollinators' flights were within 10-15 m from the mother plants, with the greatest number of flights remaining very close to the mother plant. On rare occasions we observed the powder on flowers further than 20 m, and no powder was found at distances further than 39 m from the mother plant (Ægisdóttir et al., submitted).

Seed number and SO ratio was generally not lower in the control plants compared to the outcrossing plants. This indicates that our studied *C. thyrsoides* populations do not suffer from pollen limitation but insect pollinated plant species living in small and isolated populations may show reduced reproductive output due to

pollinator limitation (Olesen and Jain, 1994; Bond, 1995). Despite the isolation of the four *C. thyrsoides* populations studied here, their population sizes were not small, suggesting there is no lack of pollinators within them. Since quite many *C. thyrsoides* populations in Switzerland are, however, not only isolated but also small (Ægisdóttir et al., submitted), we cannot exclude the possibility that pollen limitation is problematic with respect to reproduction in some *C. thyrsoides* populations.

Despite of crossing the plants with pollen from distant populations, they did neither set more seeds nor germinated better than plants that were pollinated with pollen from within the same population. This indicates that there was no hidden inbreeding depression within our studied populations. Although outbreeding depression is likely to become larger with an increased geographical or genetic distance between individual plants (Waser and Price, 1994; Montalvo and Ellstrand, 2001), we did also not find evidence for an outbreeding depression in *C. thyrsoides* since plants pollinated with long-distance pollen set as many seeds and germinated as well as plants that had been pollinated with control pollen. We can, however, not rule out the possibility that an hidden inbreeding depression or outbreeding depression would have been detectible in later life history traits of the species since the importance of measuring both early and late life history traits in plants in order to find evidence of in- or outbreeding depression has been previously stated (Waser and Price, 1994; Edmands and Timmerman, 2003). Although measuring both early and late traits in *C. thyrsoides* would have been desirable, we still found supporting evidence for inbreeding depression as well as an optimal outcrossing distance of 10 m in the study. This result is consistent with observations for many other outcrossing species which express much of their inbreeding depression early in their life-cycle, e.g. during seed production (Husband and Schemske, 1996).

The number of seeds produced per capsule differed among populations with the Furka plants setting more seeds per capsule than the other populations. Since size parameters correlated well with the number of seeds produced in a plant, the higher number of seeds produced per capsule in the Furka plants can be explained by the larger size of the Furka plants.

Conclusion

In this study we found a decreased seed set in *C. thyrsoides* flowers pollinated with pollen from their nearest neighbors (within 1 m distance). This indicates an inbreeding depression in crossings between close relatives. Moreover, we found an optimal crossing distance of 10 m. This result is in line with limited pollen flow within the populations, where the most distance pollinator flights are within 10 m from the pollen donor. Crosses between populations showed however, no signs of a hidden inbreeding depression or an outbreeding depression within our studied populations. Negative effects of detrimental mutation accumulation (which are likely to cause the inbreeding depression in *C. thyrsoides*) are expected to increase with an increasing duration of population isolation and smaller size of population (Lande, 1995). Given this, we conclude that for outcrossing species (like *C. thyrsoides*) that live in isolated habitats in the alpine landscape, inbreeding depression could be disadvantageous if populations are very small. This may explain why only sparsely distributed *C. thyrsoides* populations occur in the Alps.

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Chapter 4

Development and characterization of microsatellite DNA markers for the Alpine plant species *Campanula thyrsoides*

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Abstract

We isolated and characterized eight polymorphic microsatellite markers for the Alpine plant species *Campanula thyrsoides* (Campanulaceae). Number of alleles per locus ranged from six to 12 and the observed heterozygosity was between 0.529 and 0.900. Observed vs. expected heterozygote deficits were significantly different in one out of eight loci following Bonferroni's correction for multiple tests. We did not find evidence for linkage disequilibrium between locus pairs. The microsatellite markers are being used for the study of genetic variation and gene flow within and among populations of *C. thyrsoides* in the Swiss Alps.

Keywords: Alps, *Campanula thyrsoides*, Campanulaceae, microsatellite markers, population genetics, SSR

Campanula thyrsoides L. (Campanulaceae) is a subalpine to alpine short-lived monocarpic perennial, found on calcareous soils at about 1300-2800 m asl throughout the European Alps (Kuss *et al.* in press). The species is quite rare but locally abundant and predominantly found in pastures, extensively used hay-meadows as well as disturbed areas, such as road sides. Our studies have revealed, that *C. thyrsoides* is mainly self-incompatible and outcrossing. Its pollinators are predominantly bumblebees and other Hymenoptera. The seeds are dispersed by wind (*Ægisdóttir et al*. in press; Kuss *et al.* in press; pers. obs).

Genomic DNA was extracted from leaf samples of 10 unrelated individuals. Lyophilised leaves were homogenized and then extracted using the NucleoSpin® Plant kit (Macherey-Nagel) using buffer C1. An enriched library was made by ECOGENICS GmbH (Zürich, Switzerland) from size selected genomic DNA ligated into SAULA/SAULB-linker (Armour et al. 1994) and enriched by magnetic bead selection with biotin-labelled $(CA)_{13}$ and $(GA)_{13}$ oligonucleotide repeats (Gautschi *et al*. 2000a, b). Of 384 recombinant colonies screened, 86 gave a positive signal after hybridization. Plasmids from 48 positive clones were sequenced and primers were designed for 17 microsatellite inserts. Of these, 13 were tested for polymorphism.

We tested the primers by screening 20 individuals from a large population (> 10.000 individuals) from the Central Alps of Switzerland. We performed polymerase chain reaction (PCR) amplication in a 10-µl reaction volume containing 15 ng genomic DNA, 0.125 µM each of forward and reverse primers, 1 µl of 10x PCR buffer, 150 µM dNTP's, 0.5 U HostarTaq (Qiagen, Hombrechtikon, Switzerland). After a denaturation step at 95°C for 15 min, we performed PCR for 30 cycles: 30 s annealing at a locus-specific temperature (Table 1), 30 s at 72°C and 30 sec at 95°C. The PCR was finished with 1 min at a locus-specific temperature and a final 30-min extension step at 72°C. We performed horizontal electrophoresis on the PCR product using SEA-2000^{TM} submerged gel electrophoresis equipment (Elchrom Scientific AG, Cham, Switzerland) using Spreadex® EL-400 gels (for Campthy 3, 5) and EL-600 (for Campthy 1, 6, 13, 15) gels (Table 1). The gels were stained with ethidium bromide for 20-30 minutes. Furthermore, we took digitals images of the gels (AlphaDigiDoc and AlphaEaseFC software, Alpha Innotech Corp., San Leandro, California, USA) and analysed all clearly detectable bands by carefully verifying each gel at least 2 times.

Of the 11 tested markers, eight primer pairs gave reproducible well-scorable polymerase chain reaction (PCR) products (Table 1). We estimated expected and observed heterozygosity using GENETIX version 4.05 (Belkhir *et al*. 1996-2002). Moreover,

probability for linkage disequilibrium and deviation from Hardy-Weinberg equilibrium (HWE) were tested using the Markov chain method (Guo & Thompson 1992) in GENEPOP on the web, version 3.4 (Raymond & Rousset 1995) using 5000 dememorizations, 1000 batches and 5000 iterations per batch. A Bonferroni correction for multiple comparisons was applied in both cases to a significance level of $P < 0.05$.

We found no evidence for linkage disequilibrium. Observed heterozygosity were generally high and deviations from expected heterozygosity small, which is in accordance with our studies that indicate that *Campanula thyrsoides* is mainly self-incompatible and outcrossing (Ægisdóttir *et al*. in press). Only in one case, was the observed heterozygosity lower then the expected heterozygosity following Bonferroni's correction for multiple tests (Table 1). These microsatellite markers are being used for the study of genetic variation and gene flow within and among populations of *C. thyrsoides* in the Swiss Alps.

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Table 1 Forward (F) and reverse (R) (5' - 3') sequences and repeated motif and annealing temperatures in °C (Ta) for eight microsatellite loci characterized in *Campanula thyrsoides*. For each locus: Number of alleles (Na), allelic size range in bp (SR), observed heterozygosity (H_O), expected heterozygosity (H_E) and GenBank Accession number.

Locus	Primer sequences (5'-3')	Repeat motif	Ta (°C)	Na	SR	H_O	H_E	Accession No.
Campthy 1	F: CTGCTAGGCTATGCGAGTGTTC	$(CA)_{16}$	56	7	153-176	0.778	0.821	EF371506
	R: TCTGAATTTGTTGAGAATCTTTTTG							
Campthy 3	F: AAAGTTTGATTCCAAGGTGCTC	$(CA)_{13}$	56	6	130-155	0.737	0.680	EF371507
	R: AAAATAATTCCAGGGACGGAGT							
Campthy 5	F: CCAGCGACGCTTTAGTTATTGT	$(GT)_{20}$	56	8	93-127	0.900	0.775	EF371508
	R: CAAATATAAAGGGGAAGTTACTTATCA							
Campthy 6	F: ACAACCTCGAACCAATTTTCAG	$(CA)_{17}$	56	66	151-164	0.722	0.716	EF371509
	R: CAATTGGGGTCTAACCATTCAC							
Campthy 9	F: AATGTCCATGGTGTGGTGAAC	$(CA)_{24}$	56	7	162-192	0.778	0.809	EF371510
	R: CCATTCAAAGCCGCAGTATTAG							
Campthy 12	F: TTAATCAAATCGGCGATAATGG	$(GA)_{18}$	56	11	131-174	0.765†	0.896	EF371511
	R: CTCTCTAAGTTTTAATTGAACTGGTG							
Campthy 13	F: TCGTGTTTAGTTGCCTGATTTG	$(GA)_{30}$	60	12	166-203	0.529‡	0.850	EF371512
	R: GAGGTTTGAAAAGGTTGTCTGG							
Campthy 15	F: TTCTCCGATATATTCGCCTACC	$(CT)_{21}$	60	10	158-190	0.688	0.799	EF371513
	R: GAGCGAAAAGATATGAAGAAATTTAAG							

†, significant deviation from Hardy–Weinberg equilibrium at $P = 0.05$; ‡, significant deviation from Hardy–Weinberg equilibrium at $P = 0.00625$ following Bonferroni's correction for multiple tests.

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Chapter 5

High genetic diversity and moderate population differentiation despite natural fragmentation in a rare monocarpic alpine plant

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submitted

Abstract

• *Background and Aims* Genetic diversity and genetic differentiation within and among alpine plant populations can be greatly influenced by the strong natural fragmentation of their habitats. *Campanula thyrsoides* is a rare Alpine monocarpic perennial, occurring in isolated populations on calcareous soils and with highly limited seed dispersal capacity. The aim of this study was to investigate if the natural fragmentation of habitats has led to strong genetic differentiation and restricted gene flow among populations of *C. thyrsoides* resulting in a pronounced geographic structure.

• *Methods* We analyzed molecular diversity of 736 plants from 32 populations in the Swiss Alps using 5 polymorphic microsatellite loci and estimated pollen flow using fluorescent powder. We used individual-based Bayesian approaches to examine population structure.

• *Key Results* We found high within-population genetic diversity (H_E = 0.76), and a relatively low inbreeding coefficient (F_{IS} = 0.022). Genetic differentiation among populations was moderate (F_{ST} = 0.103) with a significant isolation-by-distance relationship (R^2 = 0.39, P < 0.001) and a significant geographic substructure coinciding with proposed postglacial migration patterns. Altitudinal location and size of populations did not influence molecular variation and direct measures of pollen flow revealed that insect-mediated pollen dispersal was restricted to short distances within a population.

• *Conclusions* Although the fragmented alpine landscape clearly restricted gene flow among the populations, the species did not exhibit as strong among-population differentiation as expected for a monocarpic species with very limiting seed dispersal capacities. This suggests a greater recent gene flow among populations than expected from direct observation or a limited depletion of genetic relatedness since range expansion after the last glaciation.

Key words: alpine plant, *Campanula thyrsoides*, genetic diversity, gene flow, genetic differentiation, glacial history, microsatellites, monocarpy, natural fragmentation, SSR

INTRODUCTION

Alpine landscapes are commonly described as strongly fragmented, with pronounced environmental gradients and heterogeneous topography. These landscape characteristics can greatly affect the life of alpine plants (Körner, 2003). Although plants exchange genes among populations through movement of both pollen and seeds, only dispersal via seeds directly affects colonization of new populations (Colas *et al.,* 1997; Cain *et al.,* 2000; Ghazoul, 2005). The geographical barriers of alpine landscapes demarcate the plants' habitats and consequently limit gene flow among plant populations and colonization opportunities of new sites (Cain *et al.,* 2000, Theurillat and Guisan, 2001). Restricted gene flow together with stronger selection pressures at higher altitudes could make within-population genetic diversity of alpine plants lower and genetic differentiation higher, compared to plants of lower altitudes and less fragmented landscapes. However, the opposite may occur (high within-population diversity at higher altitudes) due to the high environmental heterogeneity over small distances and high temporal variation of alpine habitats (Till-Bottraud and Gaudeul, 2002).

Genetic diversity and differentiation can also be influenced by several other factors, such as population size and mating system. Population size can affect random changes in allele frequencies (genetic drift) within populations by enhancing it in smaller populations, which could lead to the extinction of alleles and loss of genetic variability (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Young *et al.,* 1996; Lowe *et al.,* 2004). The impact of reproductive systems on genetic diversity in plant populations can be large. Outcrossing species usually have a high within-population diversity and low population differentiation, whereas selfing species often have low within population diversity and high differentiation among populations (Loveless and Hamrick, 1984; Hamrick and Godt, 1996, 1997; Booy *et al.,* 2000; Till-Bottraud and Gaudeul, 2002; Nybom, 2004).

In recent years, alpine ecosystems have repeatedly been put in the spotlight due to their vulnerability to global climate change. Potential ecological impacts of ongoing climate change on alpine plant communities include upward migration of species and vegetation shifts, which could lower available surface area and affect the plants' growing conditions (Grabherr *et al*., 1994; Guisan and Theurillat, 2000; Theurillat and Guisan, 2001). In that context, knowledge on the genetic variability in alpine plants has become particularly important since high genetic diversity is considered essential for plants to keep pace with the changing environment (Lande and Shannon, 1996; Booy *et al.,* 2000; Theurillat and Guisan, 2001; Till-Bottraud and Gaudeul, 2002).

Molecular studies can also provide valuable information on past biogeographical processes especially since macrofossils of herbaceous alpine plants are scarce or completely absent (Schönswetter *et al*., 2005 and references therein). Numerous molecular phylogeographic studies with a focus on glacial history and potential refugia for Alpine plants in the European Alps have been published over the last years (e.g. Stehlik, 2003; Tribsch and Schönswetter, 2003; Hewitt, 2004) and a recent synthesis demonstrated that glacial refugia were located along the border of the European Alps and in the central Alps area (Schönswetter *et al.,* 2005). Molecular data showing genetic subdivisions in species' ranges might thus provide signals of past biogeographical processes, such as glacial refugia (Comes and Kadereit, 1998; Stehlik, 2003; Tribsch and Schönswetter, 2003).

With the aim of understanding the consequences of natural fragmentation and isolation of alpine landscapes on plants as well as looking for signals of past biogeograpical processes, we studied the genetic diversity, gene flow and geographical structure of the rare Alpine plant species *Campanula thyrsoides*. Seed dispersal capacity of *C. thyrsoides* is highly limited, and the species only grows on carbonate bearing soils in the Alps and adjacent mountain ranges (Aeschimann *et al*., 2005; Kuss *et al*., in press). Despite occurring in isolated populations in a fragmented landscape where self-compatibility should be favored to ensure sexual reproduction (i.e. Baker's law; Baker, 1955, 1967), our accompanying study on the breeding system of *C. thyrsoides* showed that the species is mostly outcrossing (Ægisdóttir *et al*., 2007a). An important characteristic of *C. thyrsoides,* and the main reason for its inclusion in this study, is that it is monocarpic. Rosette-forming monocarpic plants with terminal inflorences (like *C. thyrsoides*) are quite rare amidst the temperate alpine flora (for the Alps, see Aeschimann *et al*., 2005) and are more commonly found in subtropical and tropical mountain systems (Smith and Young, 1987; Young and Augspurger, 1991). Monocarpy reduces flowering population density and consequently limits mating possibilites. The shorter generation time of monocarpic species in combination with a lower population density could lead to a reduction in the effective population size, which should promote population differentiation (Loveless and Hamrick, 1984; Vitalis *et al.,* 2004).

Here, we studied the genetic diversity and gene flow within and among 32 populations of *C. thyrsoides* covering both a large geographical and altitudinal range and using a combination of microsatellite markers and field-based estimators of pollen dispersal distances. Furthermore, we used individually-based Bayesian approaches to observe geographical structure and first generation migrants in our dataset. These approaches allow inference of geographical structure and gene flow with greater precision than alternative approaches that

rely upon idealized population models and summary statistics (Pritchard *et al.*, 2000; Piry *et al.,* 2004). Previously, we screened the same leaf material system using Randomly Amplified Polymorphic DNA (RAPD) markers and we found a moderately high within-population diversity that was not influenced by population size and altitude. Population relatedness showed a clear geographic structuring between populations in the western part of Switzerland and the central and eastern part of Switzerland (Kuss, 2006). In contrast to RAPD markers, which are dominant, microsatellites are co-dominant that enables a straightforward calculation of allele frequencies, Hardy-Weinberg proportion, and the inbreeding coefficient (F_{IS}). Another advantage to the use of microsatellites is their selectively neutral and highly polymorphic nature, which together with co-dominance, makes them very suitable for studying gene flow and population structure (Ouborg *et al.*, 1999; Lowe *et al.*, 2004; Scribner and Pearce, 2000).

We addressed the following questions: 1) How much genetic diversity exists within populations of *C. thyrsoides* and how does it relate to population size and altitude? 2) Does the inbreeding coefficient F_{IS} indicate inbreeding within the populations? 3.) Has the natural habitat fragmentation led to strong genetic differentiation and restricted gene flow among populations of *C. thyrsoides* resulting in a pronounced geographic structure?

MATERIAL AND METHODS

Study species

Campanula thyrsoides is a subalpine to alpine plant species, found on carbonate bearing soils at an altitude of 1000-2900 meters above sea level (m asl) throughout the European Alps and adjacent mountain ranges (Lauber and Wagner, 2001; Aeschimann *et al*., 2005). *C. thyrsoides* is a diploid (2n = 34) rosette-forming monocarpic plant species which flowers once in its lifetime and subsequently dies. In the year of flowering (mean: 7.5 years; range: 3-16 years), a 10-40 cm tall inflorescence is formed that carries about 50-200 flowers in a compact spike (Kuss *et al.*, in press). *C. thyrsoides* is an outcrossing species with a gametophytic self-incompatible system, i.e., it is mostly unable to self but can mate with it's half-siblings (Ægisdóttir *et al.,* 2007a). *C. thyrsoides* has a highly limited seed dispersal capacity. Seed dispersal spectra, obtained from simulations with the software Pappus (Tackenberg, 2003), showed that most seeds (99.99%) are dispersed within 10 m of the mother plant and only about 15 seeds (out of an average seed production of 1.5 million seeds per population) appear to be dispersed over 1 km (Kuss *et al.*, in press).

Campanula thyrsoides is considered rare throughout its native range but locally abundant with occassional population sizes exceeding 50,000 individuals. It is predominantly found in pastures, hay-meadows and shows the tendency to ruderalize along road shoulders. In Switzerland it is common in the northern calcareous Alps, whilst in the central siliceous Alps, it is only found in isolated carbonate-bearing outcrops (Lauber and Wagner, 2001; Kuss *et al.*, in press) (Fig. 1).

Sampling sites

We sampled leaf material from 32 populations in Switzerland ranging from the Jura Mountains in the west to the easternmost part of the Swiss Alps (Table 1; Fig. 1). In each population we randomly sampled a constant number of individual plants (n = 23). The altitude of the sampled populations ranged from 1340 to 2430 m asl. The leaf material was dried in silica gel and stored at room temperature. We assessed population sizes either by complete census or through extrapolation of average density counts from population subsets (Table 1). We only included the total population size (vegetative and flowering plants) since we observed a considerable variation in the number of flowering *C. thyrsoides* plants among years. Nevertheless, our observation showed that flowering individuals (effective population size) were ca. 5-10 % of the total populations.

DNA extraction and PCR amplification

We extracted DNA from the silica dried leaf material using a DNeasy Plant 96 Kit (Qiagen, Hombrechtikon, Switzerland) after milling 10 mg of leaf tissue (Retsch MM300). To remove polyphenols, which may adversely affect PCR amplification, we modified the manufacturer's protocol by adding 25 mg of polyvinylpyrrolidone (Fluka, Buchs, Switzerland) to each sample in the first extraction step. We quantified the DNA using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA) spectrophotometer. Samples were screened using 7 polymorphic primer pairs of which 5 were used for the final analyses, i.e. Campthy 1, Campthy 3, Campthy 5, Campthy13, Campthy 15 (Ægisdóttir *et al.*, 2007b) in order to minimize error rate and avoid pseudo replication in the event of linkage disequilibrium (as recommended by Bonin *et al*. (2004) and Selkoe and Toonen (2006)). We performed polymerase chain reaction (PCR) amplification in a 10-µl

Table 1: Geographical location, population code, coordinates, altitude (m asl), estimated population size and sample size of 32 populations of *Campanula thyrsoides* in Switzerland.

Location	Population code	Coordinates*	Altitude (m asl)	Population size	Sample size
1. Col du Marchairux, VD	JUM	508' 900/156' 400	1440	1000	23
2. Les Amburnez, VD	JUA	507' 480/155' 100	1340	1000	23
3. Pre du Rolle, VD	JUR	508' 983/155' 652	1377	150	23
4. Pres de Four, VD	JUF	498' 400/148' 450	1430	10000	23
5. Col du Jamon, VD	JAA	564' 830/145' 050	1630	100	23
6. Col du Jamon, VD	JAC	564' 589/144' 944	1670	80	23
7. Lac du Fully, VS	FUL	574' 000/113' 200	2100	500	23
8. Trient, Les Tseppes, VS	TRI	564' 350/099' 500	2020	50	23
9. Lac du Moiry, VS	MOI	609' 932/109' 638	2266	50000	23
10. Stockhorn, BE	STO	607' 737/171' 103	1980	100	23
11. Schynige Platte, BE	SPO	636' 225/167' 625	1990	600	23
12. Schynige Platte, BE	SPU	636' 600/167' 150	1890	500	23
13. Furka, UR/VS	FUR	674' 850/158' 825	2430	30000	23
14. Unterschächen, Butzlichöpf, UR	UNB	702' 500/193' 200	1900	500	23
15. Langwies, Listboden, GR	LAL	776' 750/191' 510	2000	300	23
16. Langwies, Strassberg, GR	LAS	775' 875/190' 550	1870	7000	23
17. Langwies, Holzbüel, GR	LAH	775' 010/188' 875	1700	50	23
18. Vals, Peil, GR	VAL	735' 375/160' 425	1850	100	23
19. Safiental, GR	SAF	742' 851/174' 289	1857	50	23
20. Medels, Parjurs, GR	MED	742' 800/157' 700	1870	50	23
21. Monstein, Mäschenboden, GR	MOM	779' 668/173' 708	1961	45	23
22. Monstein, Fanexmeder, GR	MOF	780' 750/174' 910	2220	250	23
23. Parsennmeder, GR	PMA	784' 030/191' 473	1995	5000	23
24. Parsennmeder, GR	PMB	784' 478/191' 548	1910	100	23
25. Churwalden, Joch, GR	CHJ	762' 300/185' 100	1890	150	23
26. St. Antönien, GR	STA	782' 203/201' 989	1943	250	23
27. Alp Laret, GR	LAR	784' 234/153' 944	2180	300	23
28. Albula Pass, Naz, GR	NAZ	778' 193/162' 751	1755	150	23
29. Schuol, La Motta, GR	SCM	816' 400/188' 400	2142	2000	23
30. Ftan, Prui, GR	FTA	812' 505/187' 750	2100	150	23
31. Tschlin, Alp Tea, GR	TEA	828' 250/198' 250	2200	150	23
32. Tschlin, Alp Tea, GR	TEB	827' 800/198' 000	2150	200	23

* Coordinates according to the Swiss topographical maps (Bundesamt für Landestopopgraphie, Wabern, Switzerland).

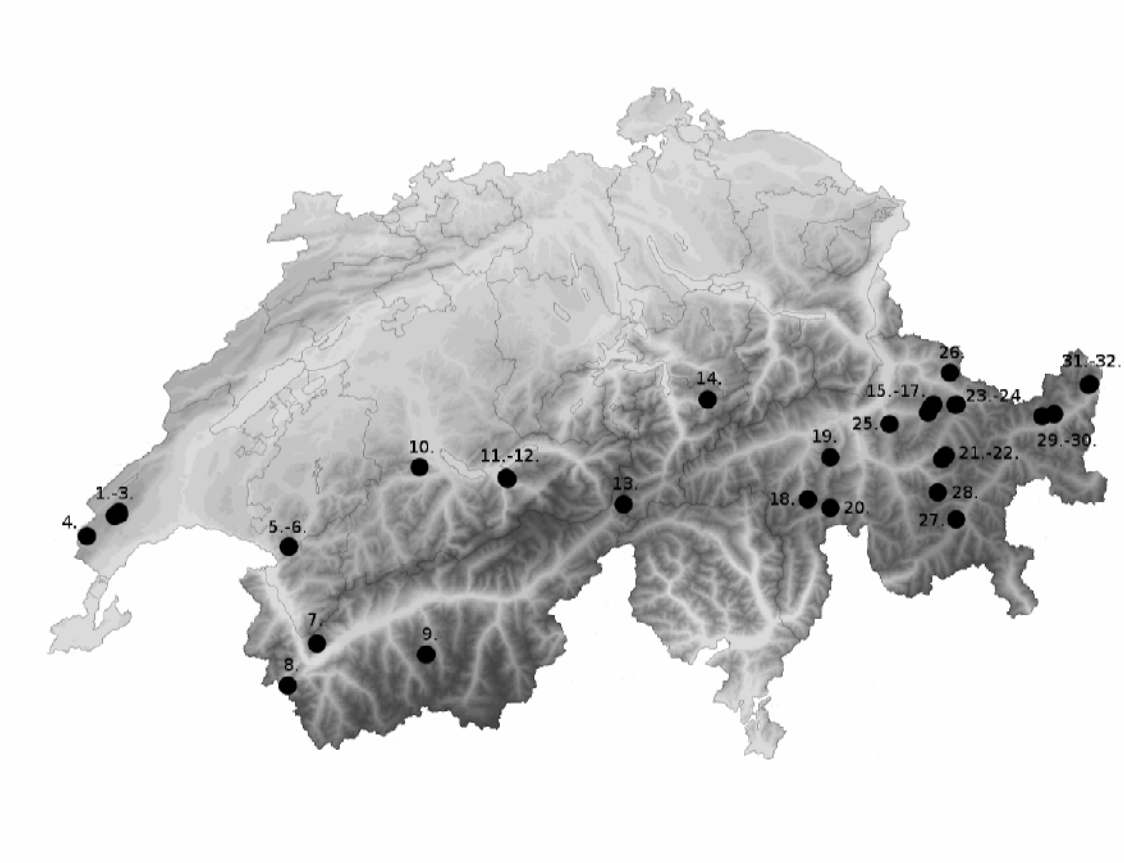

Fig. 1: Geographical location of the 32 study populations of *Campanula thyrsoides* in Switzerland (black dots). The numbers indicate the location in Table 1. The lightest gray color is at 190 m asl and the altitude increases with darker color up to about 4800 m asl).

reaction volume containing 15 ng of genomic DNA, 0.125 µM each of forward and reverse primers, 1 µl of 10x PCR buffer, 150 µM dNTP's, and 0.5 U HotstarTaq (Qiagen, Hombrechtikon, Switzerland). After a denaturation step at 95°C for 15 min, we performed PCR for 30 cycles: 30 s annealing at a locus-specific temperature, 30 s at 72°C, and 30 sec at 95°C. The PCR ended with 1 min at a locus-specific temperature, followed by a final 30-min extension step at 72°C. We performed horizontal electrophoresis of PCR products using SEA-2000^{TM} submerged gel electrophoresis equipment (Elchrom Scientific AG, Cham, Switzerland). Ethidium Bromide stained banding patterns were observed under UV light and analysed by careful manual verification of each gel at least 2 times.

We repeated all ambiguous genotyping results, in order to minimize genotyping errors, and discarded low quality or unreliable DNA samples and markers. The genotyping error rate was calculated by re-extracting and re-amplifying 39 randomly chosen plants (5.3 % of the dataset). We subsequently calculated allelic differences between those 39 plants and the

original datasets. Our error rate was estimated to be 6.1 %. The main error sources were allelic dropout and human error (sample swaps, pipetting - and scoring error).

Analysis of overall genetic variation

We calculated molecular diversity within and/or among populations with: (1) mean number of alleles per locus (N_a), (2) H_O (observed heterozygosity), (3) H_E (expected heterozygosity under Hardy-Weinberg equilibrium), (4) inbreeding coefficient (F_{IS}), and fixation index (F_{ST}) using the method of Weir and Cockerham (1984) as implemented in GENETIX version 4.05 (Belkhir *et al.*, 1996-2002). Confidence intervals (95%) were calculated for F_{IS} and F_{ST} by bootstrapping 1000 times over all loci (Weir and Cockerham, 1984). We indirectly estimated the outcrossing rates as $1-F_{IS}/1+F_{IS}$ (Ritland, 2002) and tested against 1 using a one sample t-test in R (R Development Core Team, 2004). Using GENOPOP 3.3 (Raymond and Rousset, 1995), we calculated the number of alleles per locus and assessed the probability for linkage disequilibrium and deviations from Hardy-Weinberg expectations for each pair of loci across all populations using a Fisher exact test. Confidence intervals were calculated with the Markov chain method (Guo and Thompson, 1992). A Bonferroni correction for multiple comparisons was applied in both cases.

In order to minimize missing values, individuals that had at least one clearly detectable allele but a missing value for the second allele (due to scoring uncertainty) were included in analysis based on F_{ST} but excluded from other calculations, such as H_E, Hardy-Weinberg equilibrium (HWE), and from calculations in the programs STRUCTURE and GENECLASS.

Geographical structure

In order to explore if the *C. thyrsoides* populations were confined to geographical regions (2 or 3 genepools) as found when using the RAPD markers, we used a model-based clustering method implemented in STRUCTURE 1.0 (Pritchard *et al.*, 2000). To determine the optimal number of genetic clusters present in a population, STRUCTURE divides the sampled individuals into a number of clusters (K) based only on multilocus genotypic data. We conducted 20 independent runs for each K between 1 and 5 (see appendix), and plotted the results from each run with the highest likelihood value. We used the admixture model, correlated allele frequencies and burn-in period and Markov chain Monte Carlo (MCMC) iterations of 100’000 each.

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

We furthermore tested for genetic differentiation among populations and between regions (east and west), as observed by using STRUCTURE 1.0, with molecular analysis of variance (AMOVA) (Excoffier *et al*., 1992), as applied in ARLEQUIN, using 1000 bootstrap replications.

Molecular variation and populations charateristics

We assessed the effect of population size and altitude on molecular variation (H_E, F_{IS}, and N_a) by performing single linear correlations and an analysis of variance (ANOVA) with 2 main factors (population size and altitude and their interaction) using the software R (R Development Core Team, 2004).

Gene flow estimates

Gene flow was measured with direct estimates of pollen flow in the field. Pollen flow distances were measured during the summers of 2004 and 2005 on two days with clear weather conditions on the Furka Pass in the Central Swiss Alps. In the morning, pollen from all flowers on a total of 9 plants were painted with fluorescent powder of different colors (Stockhouse, 1976). Throughout the day pollinators frequented the flowers and dispersed the fluorescent powder. After sunset traces of the fluorescent powder on *C. thyrsoides* flowers were identified using UV torches. We then proceeded to count the number of visited plants and flowers/plant, and measured the distances to the initially marked plants. Moreover, we monitored and counted insects (bumblebees, small diptera spp., beetles and ants) during 5 ½ hours of observation.

In order to estimate "real-time" migration rate of *C. thyrsoides* from the genetic data, we used GENECLASS 2.0 (Piry *et al*., 2004). For each individual plant, we estimated the likelihood of being a resident (i.e. born in the sampling location) or migrant from another reference using the Bayesian method of Rannala and Mountain (1997). We ranked all individuals using likelihood test statistic $L=L_{home}/L_{max}$ where L_{home} describes the likelihood to sample an individual from its original population and L_{max} described the corresponding

highest likelihood if sampled from all population. In addition, we used Monte Carlo re-sampling algorithm with 10,000 simulated individuals following Paetkau *et al.* (2004). The principle is to approximate the distribution of multilocus genotype likelihoods for individuals belonging to a particular population. An individual is then identified as an immigrant if L is smaller than for the proportion 1-α (type I error rate) of the simulated individuals. We selected an alpha level of 0.01 to determine critical values since simulated data have shown this level to represent an appropriate balance between stringency and power (Paetkau *et al.*, 2004).

RESULTS

Overall microsatellite diversity

We genotyped 5 loci in 736 *C. thyrsoides* plants and detected 100 alleles in total and 15-28 alleles per locus with an average of 20 alleles. At the intra-populations level, the number of alleles per locus ranged from 5.60 to 10.20 with an average of 8.00 (Table 2). The observed heterozygosity (H_O) over all populations was high (mean H_O: 0.763; range: 0.645 – 0.849) and deviation from expected heterozygosity (H_E) was small (mean H_E: 0.762; range: 0.666-0.847) (Table 2). There was no significant difference in expected heterozygosity (H_E) among populations ($F = 1.72$; $P = 0.20$).

The average inbreeding coefficient was relatively low ($F_{IS} = 0.022$), with most values ranging from -0.0062 to 0.0603 on the locus level and 0.017 to 0.025 on the population level (Table 2). Analysis of per-locus values from the F_{IS} statistics identified three out of five loci that deviated significantly from HWE. Likewise, 11 out of 32 populations deviated significantly from HWE (Table 2). The outcrossing rate was high, ranging from 0.952 to 0.966 with an average of 0.958, but differed significantly from 1.0 (t-test, t = -59.5132, $P < 0.001$). We found no evidence for linkage disequilibrium between the studied loci.

Altitude (m asl) had no influence on molecular variation within populations (H_E: $R^2 = 0.04$, $P = 0.29$; F_{IS}: $R^2 = 0.02$, $P = 0.89$; N_a: $R^2 = 0.004$, $P = 0.72$). Similarly, population size had no influence on the molecular variation (H_E: $R^2 = 0.01$, $P = 0.078$; F_{IS}: $R^2 = 0.03$, $P = 0.37$; N_a: $R^2 = 0.011$, $P = 0.56$). Moreover, the ANOVA analysis revealed no significant difference between molecular variation (H_E, F_{IS} and N_a) and population charateristics (altitude, population size) (Table 3).

Genetic diversity among populations and geographical structure

We analysed the data by increasing *K* from two to five using STRUCTURE. For *K* = 2, we found a clear West-East division, with populations numbered 1-9 clustering together in Western Switzerland and populations numbered 10-32 clustering in central and eastern Switzerland (Fig. 2 above). For *K* = 3 and *K* = 4, the West-East divisions were still very clear and we also found another division with a new genepools in the east (one regional genepool for *K* = 3, and two for *K* = 4, see Fig. 2 below for *K* = 3). For *K* = 5, the populations in the West region are divided into two genepools and population in the Central-East region into 3 genepools (figure not shown).

Genetic differentiation (F_{ST}) between population pairs ranged from -0.004 to 0.241 with an average of 0.103. Of the 496 pairwise F_{ST} values, all except one were significant. Genetic differentiation between the populations correlated significantly with geographical distances (Mantel test based on pairwise F_{ST}; $R^2 = 0.39$; $P < 0.001$; Fig. 3a). When the two regions (East and West) were tested seperately, there was still a significant correlation between the geographical distances and the pairwise F_{ST} in the Eastern region ($R^2 = 0.07$; $P < 0.05$; Fig. 3b). In the Western region however, the relationship was only marginally significant ($R^2 = 0.064$; $P = 0.052$; Fig. 3c).

Analysis of molecular variance (AMOVA) showed that 86.7 % of the variation was assigned within the populations, whereas 7.91 % was assigned among populations within regions and 5.39 % was assigned between regions (Table 4).

Gene flow

Traces of fluorescent powder (corresponding pollen flow) were found 1102 times on a total of 173 plants. On average the powder was found on 6.4 flowers per plant. The frequency of observed pollen dispersal distances was highest within the first 2 meters from the marked plant, but decreased markedly with increased distance. The longest observed dispersal distance was 39 m (Fig. 4). Bumblebees were the prime flower visitor, accounting for 60% of the total visits, followed by small diptera species whose contribution measured about 30% of the total visits. Bumblebees were observed collecting a large amount of pollen from flowers and seemed to be much more efficient in dispersing pollen between flowers and individual plants than the other insect groups.

Detection of first generation migrants, using GENECLASS 2.0 showed that only 18 plants (of 736 plants; 2.4 %) had a probability value below 0.01 to originate from the population where they were sampled and were therefore considered immigrants. Half of the

migrants (n = 9) were thought to come from localities nearby (<50 km) and the other half (n = 9) was considered to have their source population further away.

Table 2: Mean number of alleles per locus (N_a), expected heterozygosity (H_E), observed heterozygosity (H_O) and inbreeding coefficent (F_{IS}) for each studied population of *Campanula thyrsoides* across 5 loci.

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

†Statistically significant deviations from Hardy-Weinberg expectations by **($p < 0.01$) and *** ($p < 0.001$)

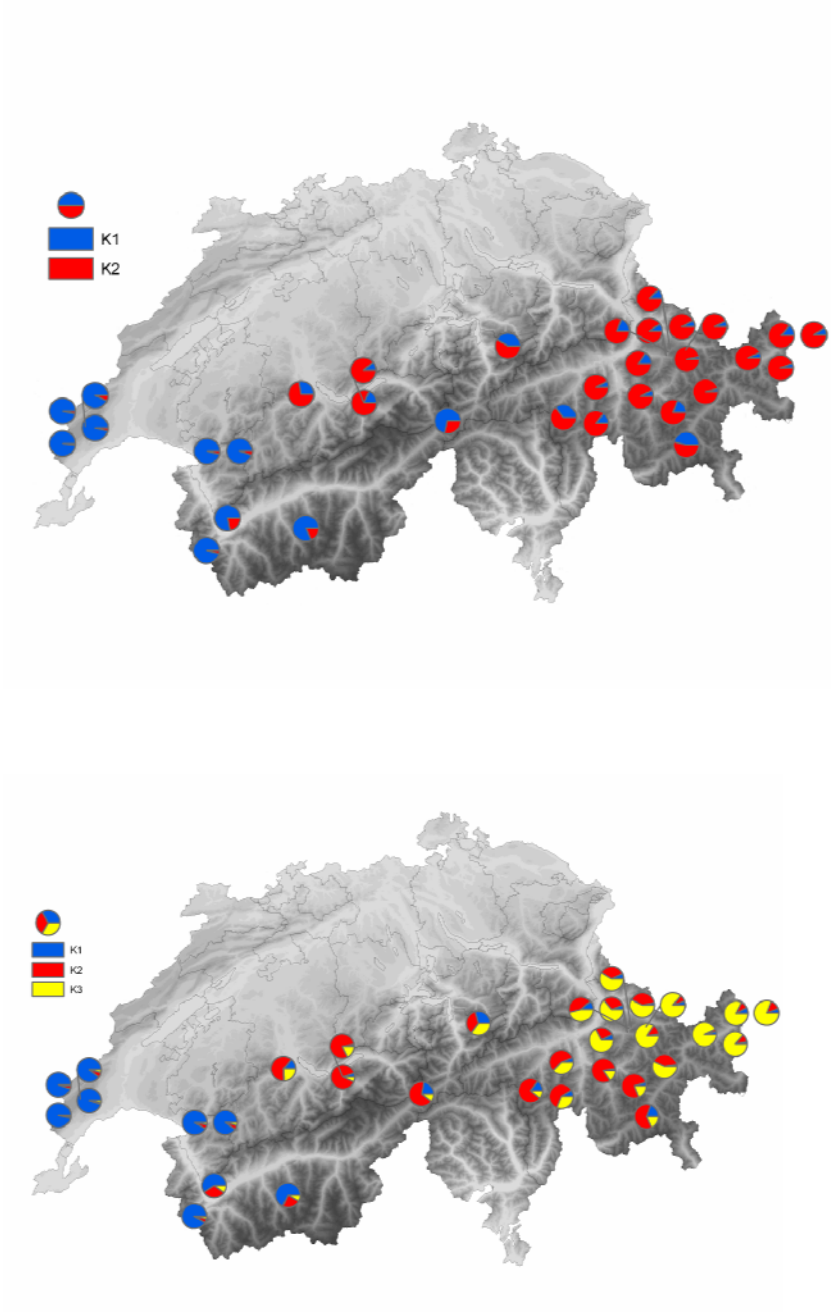

Fig. 2: Distribution of: a) genepool 1 (K1) and genepool 2 (K2) (above), and b) genepool 1 (K1), genepool 2 (K2) and genepool 3 (K3) (below) among 32 populations of *Campanula thyrsoides* in Switzerland.

Table 3: Analysis of variance (ANOVA) of the effect of altitude, population size and their interaction on expected heterozygosity H_E, inbreeding coefficient F_{IS}, and mean number of alleles per locus N_a.

		H_E	F_{IS}	N_a
Source of variation	**d.f.**	**p**	**P**	**p**
Altitude	1	0.25	0.89	0.71
Population size	1	0.12	0.38	0.63
Altitude x Population size	1	0.055	0.71	0.16
Residual	28			

d.f., degrees of freedom; p, level of significance.

Table 4: Summary of analysis of molecular variance (AMOVA) of 32 populations of *Campanula thyrsoides* grouped in two regions (West vs. East).

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

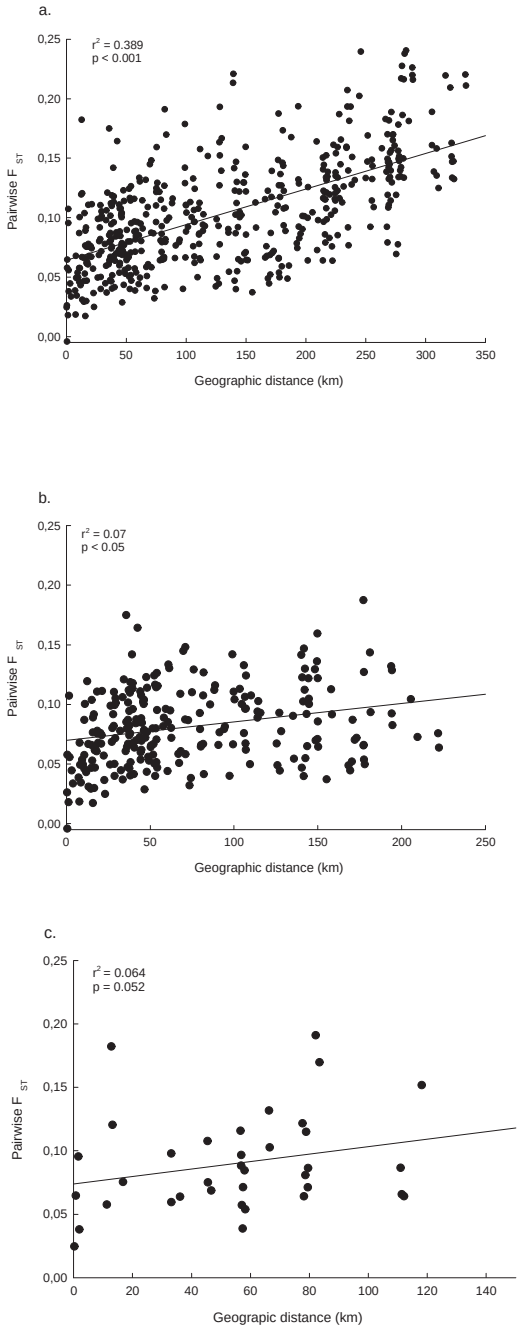

Fig. 3: Correlation of genetic (pairwise F_{ST}) and geographical distances (km) between populations of *Campanula thyrsoides*: a) all studied populations,b) populations in region 1 (no. 10-32), and c) populations in regions 2 (no. 1-9).

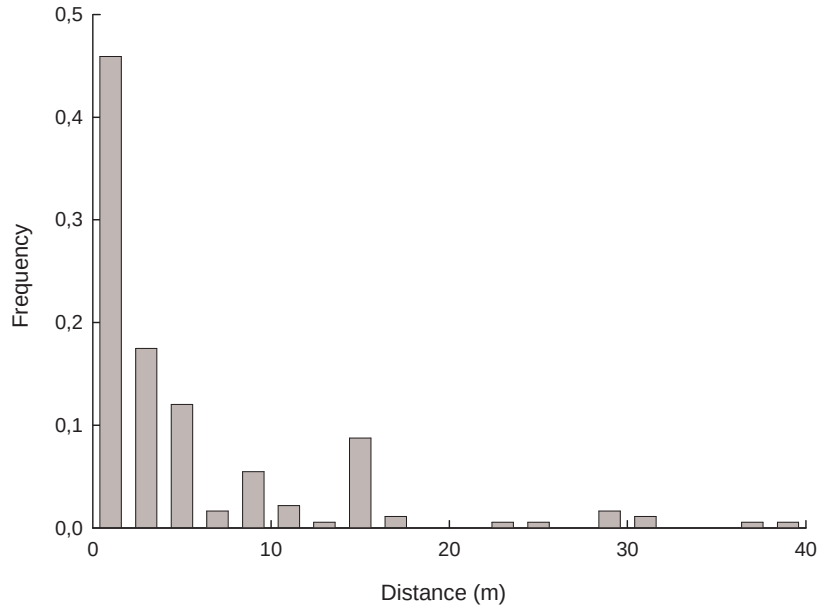

Fig. 4: Frequency of pollinator flight distances (meters) between *Campanula thyrsoides* plants on the Furka Pass in the Swiss Alps, summer 2004 and 2005 ($N = 173$).

DISCUSSION

Genetic diversity within populations

We found higher levels of genetic diversity within populations of *C. thyrsoides* (expected heterozygosity; $H_E = 0.762$) than many other microsatellite-based plant studies (e.g. Sun and Salomon, 2003; Mengoni *et al.*, 2000; Galeuchet *et al*., 2005; Gao, 2005). A review by Nybom (2004), reported for microsatellite studies an average H_E of 0.55 for short-lived perennials and H_E of 0.65 for outcrossing plant species.

Population size did not affect molecular diversity in *C. thyrsoides* (Table 3) despite an existing theory's prediction that small populations might lose genetic variation due to genetic drift, founder effects, and population bottlenecks (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Young *et al*., 1996; Lowe *et al*., 2004). Although many studies report a positive relationship between genetic variation and population size (e.g. Frankham, 1996 and references therin), the so far few studies on Alpine plant species that include comparisons of genetic diversity and population sizes could not detect any correlation, such as *Geum reptans* (Pluess and Stöcklin, 2004) and *Saxifraga oppositifolia* (Gugerli *et al.*, 1999). The same has been recorded for some none-alpine species, e.g. *Acacia anomala* (Coates, 1988), *Eucalyptus*

parvifolia (Prober *et al*., 1990) and *E. pendens* (Moran and Hooper, 1987), see also Ellstrand and Elam (1993).

Due to the harsh environmental conditions, plants are often believed to be exposed to stronger selection at higher altitude. This could lead to lower genetic diversity within plant populations in Alpine environments compared to those populations at lower altitudes (Till-Bottraud and Gaudeul, 2002). In agreement with the few studies on alpine plants that have directly studied the effect of altitude on genetic diversity in plants (Gugerli *et al*., 1999; Pluess and Stöcklin, 2004; Zhao *et al*., 2006; Kuss *et al*., 2006), we did not find evidence for a lower genetic diversity with increasing altitude. If selection pressure is stronger at higher altitudes, why did we not find a reduced genetic diversity with increasing altitude? A possible explanation is that high environmental heterogeneity over small distances and high temporal variation of Alpine habitats cause the opposite effect, i.e., high genetic diversity within populations at higher altitudes, i.e. genetically diverse plants are better capable to cope with the alpine environment (Till-Bottraud and Gaudeul, 2002).

The high within-population diversity in *C. thyrsoides* is also likely to be influenced by some life history traits of the species, especially the type of breeding system, which has been shown to strongly affect the distribution and magnitude of genetic diversity in plant populations. Outcrossing plant species tend to have higher genetic variation within populations, whereas populations of selfing species or species with a mixed mating system are often genetically less variable (Loveless and Hamrick, 1984; Hamrick and Godt, 1996, 1997; Booy *et al*., 2000; Till-Bottraud and Gaudeul, 2002; Nybom, 2004; Duminil *et al*., 2007). Since *C. thyrsoides* is predominantly outbreeding (Ægisdóttir *et al*., 2007a; Ægisdóttir *et al.*, submitted) with a high outcrossing rate (average: 0.96) and low inbreeding coefficient (F_{IS} = 0.022), we suggest that the breeding system of *C. thyrsoides* plays an important role in driving the high within-population diversity in this species. Furthermore, the high number of traces of fluorescent powder observed in the field (1102 times on 173 plants) with occasional flight distances of 39 meters indicate that pollinators (especially bumblebees) contribute to the high genetic variation within populations.

Heterozygote deficiency

Whilst the inbreeding coefficent (F_{IS}) was low in all populations (average F_{IS} = 0.022), most values were positive and 11 populations out of 32 significantly deviated from HWE. Heterozygote deficiency in a population can occur as a result of inbreeding, selection against heterozygotes, the Wahlund effect (reduction of heterozygosity in a population caused

by subpopulation structure) or the presence of null alleles (Sun and Salomon, 2003 and references therein). We consider some of these explanations unlikely (e.g: the Wahlund effect, presence of null alleles), although not all of these explanations can be definitively excluded. Despite *C. thyrsoides* being an outbreeding species, its ability to mate with it's half-siblings (Ægisdóttir *et al.*, 2007a) allows for the possibility of some inbreeding. In a field experiment testing for a pollination-distance effect in *C. thyrsoides*, we found that seed set was lower in plants pollinated with a close by pollen donor compared to those with donor plants from further away. This suggests an inbreeding depression which is probably a consequence of mating between half-sisters (Ægisdóttir *et al*., submitted). Inbreeding due to half-sister mating, is thus the most likely explanation for the deviance from random mating (HWE) in our study.

Genetic differentiation among populations

A review on among-population genetic diversity (F_{ST}) in plants using microsatellites (Nybom, 2004), showed contrasting average F_{ST} values for short-lived perennials (F_{ST} = 0.31), outcrossing species (F_{ST} = 0.22) and for species with wind dispersed seeds (F_{ST} = 0.13). It has been argued that the high levels of within-population heterozygosity usually found with microsatellites result in much lower differentiation estimates (Hedrick, 1999). Since we observed high within-population diversity in our study (H_E = 0.762), we consider that a F_{ST} value of 0.103 represents a moderate genetic differentiation. A clearly low genetic differentiation is likely to resemble the value observed for *Lychnis flos-cuculi* in Switzerland (F_{ST} = 0.022; Galeuchet *et al.*, 2005). Thus the observed differentiaton among *C. thyrsoides* populations indicates restricted gene flow among populations as would be expected for a species living in a fragmented landscape (Cain *et al*., 2000; Theurillat and Guisan, 2001). Moreover, only 18 individuals could be classified as first generation migrants with a probability value below 0.01, indicating restricted gene flow among populations. Nevertheless, the genetic differentiation was also not particularly high, especially when the life-history of the species (being monocarpic, which should promote genetic differentiation; Loveless and Hamrick, 1984) and its highly limiting dispersal capacity is taken into account.

Furthermore, the correlation between geographic distance and genetic distance (Fig. 3a), suggests that gene flow occurs mostly among close and adjacent populations (Wright, 1943). A stronger divergence among populations as geographic distance increases should be particularly prominent in the alpine landscape as a result of fragmentation and isolation of their habitats (Till-Bottraud and Gaudeul, 2002).

Geographical structure

The clear geographical boundary existing between populations in Western Switzerland and populations in Central-Eastern Switzerland (Fig. 2a), was already found with RAPD profiles (Kuss, 2006), and remained after increasing the number of genepools in STRUCTURE to $K = 3$ (Fig. 2b) and $K = 4$ (figure not shown). AMOVA indicated that 5.39% of the genetic diversity was found among regions (Table 4). Since the observed West-East division in our populations did not correspond to the geographical division between the Jura mountains in the West and the Alps in the East, we consider it most likely that this genetic boundary arose as a result of post glacial colonization history, as it corresponds to the glacial refugia map for mountain plants in the European Alps proposed by Schönswetter *et al*. (2005).

Using STRUCTURE, we identified another genepool in the eastern most populations (Fig. 2b) using $K = 3$. Using four genepools ($K = 4$) we, moreover, identified one more genepool that was regional in the eastern part of Switzerland (figure not shown). These genepools may have originated from a refugias for mountain plants of calcareous bedrock, which were located in Central and Eastern Switzerland (Schönswetter *et al.*, 2005).

Whilst measuring pollen movement with fluorescent powder in the field gave us an interesting view of the pollinator's role with regard to gene flow in plants, a clear disadvantage of this method was the difficulty in detecting long-distance dispersal. The software PAPPUS, which has been used to estimate seed flow in *C. thyrsoides* (Kuss *et al*., in press), is however, considered superior for predicting long-distance dispersal via seeds because of the incorporation of turbulence (especially thermal updrafts) and the consideration of topographic effects (Tackenberg, 2003). Despite, that PAPPUS estimated gene flow via seeds to be very low in *C. thyrsoides*, and the fact that we only detected 18 migrants with a probability value below 0.01 using GENECLASS, we found indication that some long-distance dispersal occurs since half of the GENECLASS migrants (n = 9) were thought to arrive from locations more than 50 km away from their sampling location. Secondary seed dispersal in *C. thyrsoides*, e.g. via birds or other animals, could possibly contribute for this long-distance dispersal. The role of that is, however, not known.

It is not possible to predict the timescale of the gene flow inferred on the basis of the moderate F_{ST} value or an isolation by distance pattern as observed in our study. Are we dealing with a greater contemporary gene flow than we were able to measure in the field (pollen and seed flow) or a historical gene flow which has had insufficient time to allow for

high genetic differentiation (eg: since the last glacial period)? Because of the poor dispersal capacity and the isolation of suitable habitats, we find it most likely that the genetic pattern found in *C. thyrsoides* is at least partly reflecting a historical gene flow over a timescale of several hundred years or thousands of years. Possibly, this gene flow has occurred since the end of the last glacial period (10,000 years ago) when the plants species invaded Switzerland from at least two different glacial refugia as indicated by the geographical pattern observed with STRUCTURE (Fig. 2).

Conclusions

We report a high within-population genetic diversity for the rare monocarpic perennial *Campanula thyrsoides* in the Swiss Alps, which can best be explained by the outbreeding behaviour of the species. Despite living in isolated populations of different sizes and at contrasting altitudes in the fragmented alpine landscape, neither fragmentation as such nor population size or altitude negatively affected genetic variability within the studied populations.

The fragmented landscape of the Alps clearly restricts gene flow among populations of *C. thyrsoides*, which was demonstrated by a significant isolation by distance relationship and moderate genetic differentiation. Nevertheless, we observed a lower genetic differentiation than would be expected in a monocapic perennial that lives in isolated populations and has very poor seed dispersal capacity. In addition, we found a clear geographical boundary among populations in Western versus Central/Eastern regions of Switzerland, which corresponds well with the post glacial colonization history of many other alpine plants. This suggests either a greater recent gene flow between populations than expected, or a historical gene flow which has had insufficient time to allow genetic differentiation to occur (e.g.: since the last glacial period).

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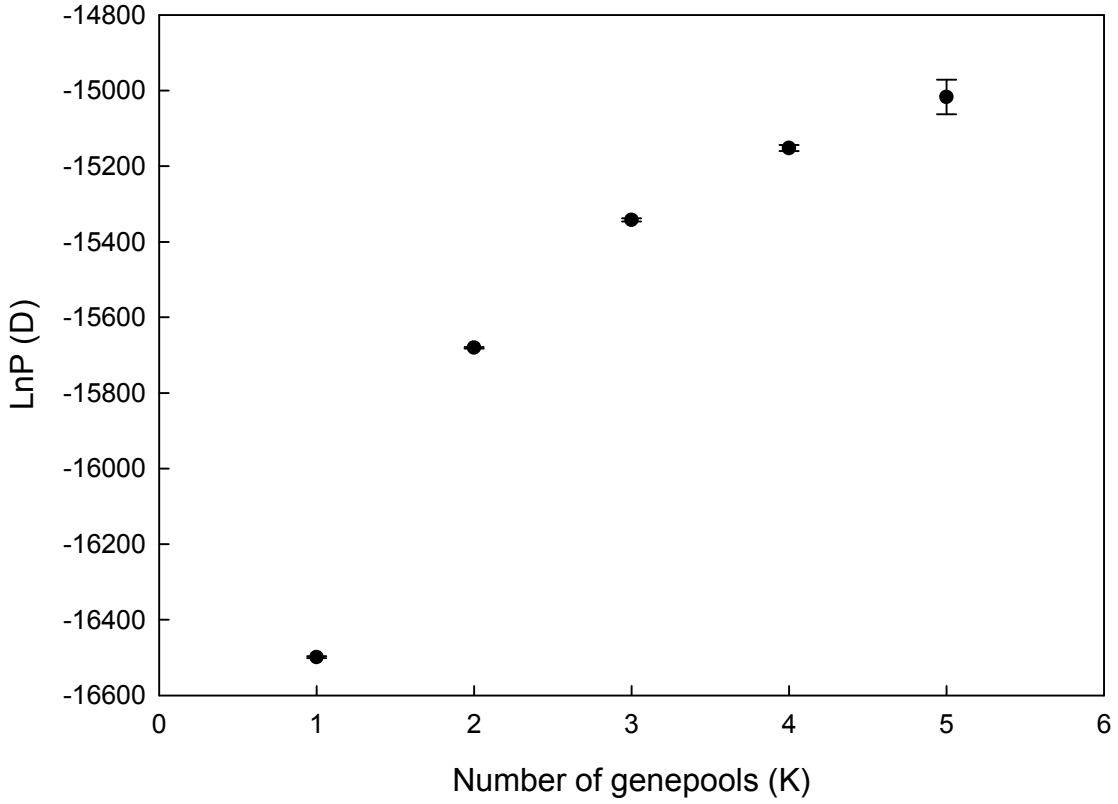

Appendix: Mean values of 20 runs (± standard deviation) of Ln probability of data (LnP(D)) for K from 1-5 as implemented in STRUCTURE on *Campanula thyrsoides.*

Chapter 6

Spatial isolation and genetic differentiation in naturally fragmented alpine plant populations

Patrick Kuss, Andrea R. Pluess, Hafdís Hanna Ægisdóttir & Jürg Stöcklin

Abstract

The effect of landscape fragmentation on the genetic diversity and adaptive potential of plant populations is a major issue in conservation biology. Here, we investigate molecular patterns of three Alpine plants and ask whether spatial isolation has led to high levels of population differentiation, increasing over distance, and to a decrease of within-population variability. For all species we found a significant isolation-by-distance relationship but only a moderately high differentiation among populations (Φ_{st}: 14.8 %, 16.8 %, 22.7 %, respectively). Within-population diversity was not reduced in comparison to lowland species (H_e: 0.19-0.21, P_p: 62-75 %) and even small populations with less than 50 reproductive individuals contained high levels of genetic diversity. We further found no indication that a high long-distance dispersal potential enhances genetic connectivity among populations. Gene flow seems to have a strong stochastic component causing large dissimilarity between population pairs irrespective of the spatial distance. Our results suggest that other life-history traits, especially the breeding system, may play an important role in genetic diversity partitioning. We conclude that spatial isolation in the alpine environment has a strong influence on population relatedness but that a number of factors can considerably influence the strength of this relationship.

Keywords: *Campanula thyrsoides*; *Epilobium fleischeri*; *Geum reptans*; isolation by distance; life-history traits; molecular diversity; RAPDs; Swiss Alps.

Introduction

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

In general, habitat fragmentation and the resulting decline in population size can have a multitude of effects, such as erosion of genetic variation, increased random genetic drift or elevetated inbreeding, which can all enhance the risk of extinction (e.g. Gilpin and Soulé, 1986; Young et al., 1996; Frankham and Ralls, 1998). An intuitive consequence of spatial isolation is a reduced genetic connectivity between populations which leads to stronger dissimilarity of population pairs with increasing distances, generally referred to as 'isolation-by-distance' (IBD, Wright, 1943). However, the magnitude and significance of IBD patterns is often considered to be a result of a number of additional factors, such as physical barriers, dispersal ability, effective population size, maximim geographic sampling distance or time since colonization (e.g. Garnier et al., 2004; Crispo and Hendry, 2005) so that general predictions are difficult to make. Further, irrespective of habitat fragmentation, there is still considerable uncertainty about the relative influence of specific environmental constraints, the Quaternary history and life-history traits on the genetic diversity of a given species. Since

molecular markers have different resolutions and modes of inheritance (Lowe et al., 2004) they tend to emphasize different factors. In this respect, reviews of nuclear marker studies of predominantly lowland species showed that long-lived, outcrossing, late successional plant species retain the greatest share of their genetic variability within populations, while for annual, selfing and/or early successional taxa, a high percentage of genetic diversity is found among populations (Hamrick and Godt, 1989; Nybom and Bartish, 2000; Nybom, 2004). Similar results have been shown for alpine species (Till-Bottraud and Gaudeul, 2002) but, with only a few studies available, an effect of harsh alpine habitats cannot be ruled out. On the other hand, a meta-analysis of cpDNA studies found little influence of life-history traits on genetic diversity, but evidence for glaciation-derived patterns (Aguinagalde et al., 2005).

Here, we study and compare genetic diversity and differentiation of three representative alpine perennial plant species. Given the complex interactions involved in the creation of molecular patterns as outlined above, we took particular care to standardize as many parameters as possible in order to minimize known biases (Nybom and Bartish, 2000; Lowe et al., 2004). We standardized the number of populations, individuals, RAPD primers as well as loci for each species and further restricted the data analysis to the same maximum geographic distance within a single prominent area of post-glacial migration. We also decided to re-analize results of a previous study on the alpine *Geum reptans* (Pluess and Stöcklin, 2004a) with two new investigations on *Epilobium fleischeri* and *Campanula thyrsoides*. Thus, in this common framework we can considerably improve the comparability of individual patterns. Our objective is to elucidate the effect of natural fragmentation on the genetic diversity of alpine plant species in which spatial isolation can be assumed to have existed for centuries or millenia. In particular (1) we expect genetic population differentiation to be high and significantly increasing with increasing distances. Since our study species differ particularly with respect to long-distance seed dispersal, (2) we expect relatively lower genetic differentiation and the least pronounced isolation-by-distance pattern for species with morphological adaptations to seed dispersal compared to plants lacking those functional structures. (3) We further investigate levels of within-population diversity and expect a significant decrease of diversity with decreasing population sizes.

Table 1. Life-history traits of three Alpine plant species.

Species	Habitat[a]	Altitudinal range (m)[a]	Breeding system	Seeds/pop (Mio)	Dispersal (%>1km)[g]	Pollen flow (m)	Clonality	Ramet age (yrs)
Epilobium fleischeri	Glacier forelands, river bank	1000 - 2700	Mixed[b]	4.5[e]	Wind 0.5	Insects 30[h]	Rhizomatous[b]	30[e]
Geum reptans	Glacier forelands, blockfields	1950 - 3500	Outcrossing[c]	10[f]	Wind 0.005[f]	Insects 30[f]	Stolons[f]	30[f]
Campanula thyrsoides	Alpine meadows, pastures	1300 - 2800	Outcrossing[d]	1.5[d]	Wind 0.001	Insects 39[d]	Non-clonal[a]	3-16[i]

[a] Hegi (1995), [b] Theurillat (1979) [c] Rusterholz et al. (1993), [d] Ægisdóttir (in prep.), [e] Stöcklin and Bäumler (1996) , [f] Pluess and Stöcklin (2004a), [g] Tackenberg (Uni Regensburg, Germany, personal comm.), [h] Stöcklin (unpublished), [i] Kuss (in prep.)

Materials and methods

The plant species

Epilobium fleischeri Hochst. (Onagraceae), *Geum reptans* L. (Rosaceae), and *Campanula thyrsoides* L. (Campanulaceae) are widespread alpine plant species native to the European Alps and, partly, to adjacent mountain ranges in the East (Carpatians and Dinarians) and North-West (Jura; see Hegi, 1995). Throughout their ranges, plants are rare but locally abundant with population sizes ranging from a few hundred to over 50000 individuals. *E. fleischeri* and *G. reptans* are characeristic plants of glacier forelands appearing within few years after ice retreat. *C. thyrsoides* is found in mesic alpine meadows on calcareous soil. The species investigated differ with respect to several important life-history traits that potentially and differentially influence genetic-diversity partitioning within and among populations (Table 1). Life-history information are cumulated from a number of literature sources or from additional unpublished experiments and observations made by the authors. Data for long-distance seed dispersal was generated with the software PAPPUS implementing an alpine wind data set and the terminal velocity of the seeds (Tackenberg, 2003). Pollen flow observations are minimum distance estimates derived from flower to flower fluorescent powder transport by mainly bumble bees (*E. fleischeri, C. thyrsoides*) or flies (*G. reptans*) as described in Pluess and Stöcklin (2004a). We conducted pollinator exclosure and manual crossing experiments for the species to estimate individual self-compatibility as well as seed set under different pollination events. Ramet age estimates stem from herb chronology studies

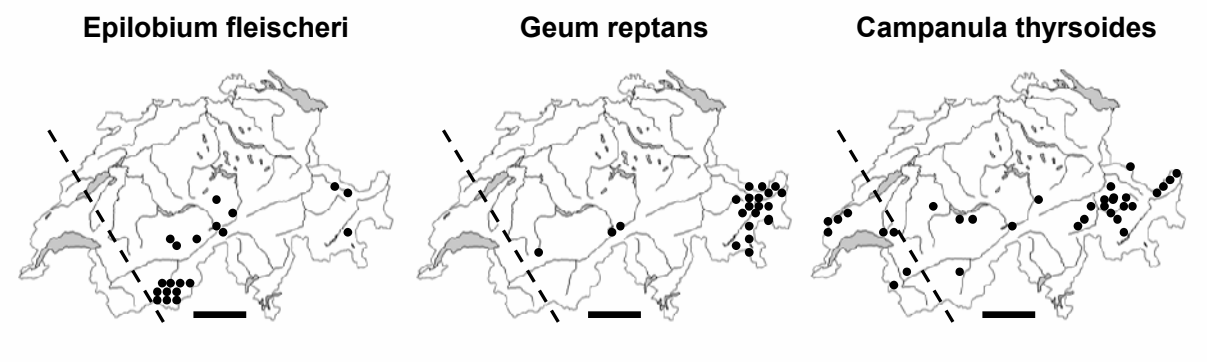

Fig. 1: Geographic distribution of the studied populations of *Epilobium fleischeri*, *Geum reptans* and *Campanula thyrsoides* in the Swiss Alps and the Jura Mountains. Dashed line represents approximate border line between two glacial refugia (Schönswetter et al., 2005). Bar: 50 km.

of roots with a representative number of individuals as presented in Dietz and Ullmann (1998). It would be desirable to have information on the potential genet age in the clonal species *E. fleischeri* and *G. reptans*, but investigations are still missing.

Sampling design

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

Molecular marker suitability

We tested two molecular methods that appeared promising for the application in all three study species, i.e. allozymes, RAPD. For *E. fleischeri* and *C. thyrsoides* we screened 22 and 18 isozyme-systems respectively on cellulose acetate gels in different electrophoresis buffer systems (TG, CAAPM, C). No polymorphisms were detected despite this large

quantity of enzyme systems, including enzymes that have been successfully used in other *Epilobium,* i.e. MDH, PGI (Brian Husband, University of Guelph, Canada, pers. comm.), and *Campanula* species, i.e. AAT, GPI, IDH, MDH, 6PGDH, TPI (Ægisdóttir, 2003). Our results suggest that for both species most isozymes are fixed.

RAPD-markers

In the case of *E. fleischeri* and *G. reptans* we homogenized 20 mg silica dried leaf tissue (Retsch MM2, Retsch GmbH & Co KG, Haan, Germany) and extracted DNA with a DNeasy plant mini kit (Qiagen GmbH, Hilten, Germany). DNA concentrations were measured by fluorimetry (Turner design, Sunnyvale, California, USA) with PicoGreen dsDNA quantitation reagent (Molecular Probes Inc., Eugene, California, USA). For *C. thyrsoides* we milled 10 mg silica dried leaf tissue (Retsch MM300) and extracted DNA with a DNeasy Plant 96 Kit (Qiagen). We modified the manufacturers protocol by adding 25 mg polyvinylpyrrolidone (Fluka, Buchs, Switzerland) to each sample in the first extraction steps in order to remove polyphenols that may interfere with PCR amplification. DNA quantification was done spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA). After an initial screening of up to 60 decamer primers we restricted the final analysis to 5 primers for each species (Kit A, K and P, Operon Technologies Inc., Alameda, California, USA and M-6 Microsynth, Balgach, Switzerland). We attempted to use the same 5 primers for all species but PCR products could not be obtained with this prerequisite. Therefore, we selected the primers with the highest number of reproducible polymorphic bands: *E. fleischeri* (OPA-8 [GTG ACG TAG G]; OPA-9 [GGG TAA CGC C]; OPA-12 [TCG GCG ATA G]; OPA-15 [TTC CGA ACC C]; OPP-12 [AAG GGC GAG T];), *G. reptans* (M06 [GTG GGC TGA C]; OPP-8 [ACA TCG CCC A]; OPP-9 [GTG GTC CGC A]; OPP-17 [TGA CCC GCC T]; OPP-19 [GGG AAG GAC A]), *C. thyrsoides* (OPA-7 [GAA ACG GGT G];OPA-11 [CAA TCG CCG T]; OPA-13 [CAG CAC CCA C];OPA-19 [CAA ACG TCG G]; OPP-3 [CTG ATA CGC C]). Amplifications were carried out in 25-µL reaction mixture containing 3 ng of template DNA, 100 µM dNTPs, 0.2 µmol/L primer, 1 × Taq Polymerase Buffer (*E. fleischeri* and *C. thyrsoides*: Qiagen, Hilten, Germany; *G. reptans*: Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), additional 0.5 mmol/L $MgCl_2$ for the primers OPA-12, OPP-17, OPP-19, M-6, additional 1.5 mmol/L $MgCl_2$ for the primers OPA-8, OPA-15, and 1 U Taq DNA Polymerase (*E. fleischeri* and *C. thyrsoides*: Qiagen, Hilten, Germany; *G. reptans*: Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). To assure consistency in the PCRs we kept aliquots of a

single master mix per two primers for all samples only adding primer, Taq Polymerase, and DNA before PCR. All PCRs were performed in the same thermal cycler (PTC-100, MJ Research, Inc., Watertown, Massachusetts, USA) programmed for 60 s at 93°C to denature the DNA followed by 34 cycles of 30 s at 92°C, 30 s at 37°C and 90 s at 72°C. Final extension lasted for 5 min at 72°C. Samples were kept at 4°C until further analysis. The PCR products were separated on 1.6 % agarose gels (Sea Kem LE agarose, BMA, Rockland, Maryland, USA) in 1 × TAE (Tris/Acetat/EDTA) buffer in an electrical field (170 mV). Depending on the RAPD primer gels were run between 1.75 and 2.5 h and stained with ethidium bromide for 20 min. We visualized the banding pattern under UV light and scored the presence and absence of bands within an estimated fragment length range of 450 to 2000 bp from digital images (AlphaDigiDoc and AlphaEaseFC software, Alpha Innotech Corp., San Leandro, California, USA).

To assure reproducibility and assess genotyping errors (Bonin et al., 2004), we repeated amplification at timely intervals with 12 initial screening samples, i.e. three individuals from each of four distinct populations. This was also repeatedly done for randomly chosen individuals. All amplifications contained blind samples (no DNA) or foreign DNA from different plant species (*Campanula barbata, Senecio incanus, Hypocheris uniflora*). Monomorphic bands served as references for genotype errors within and between amplification. The main source of genotyping error were 'ghost bands', faint bands that could not be scored unambiguously. In most cases repeated amplification of these individuals revealed a present band. If doubts remained, this locus was discarded totally. We further simulated the effect of artificially introduced random errors in the data sets and found no significant difference in genetic indices with artificial error rates up to 7 % (data not shown). Thus, potential misclassification of RAPD bands is counterbalanced by a high number of populations and individuals therein. The final presence/absence data matrix contained for *E. fleischeri*: 400 individuals from 20 populations, for *G. reptans*: 386 individuals from 20 populations, and for *C. thyrsoides*: 736 individuals from 32 populations (Appendix 1).

Statistical analysis

In order to achieve a comparable framework for the statistical analysis, we adjusted our data sets in several consecutive steps to avoid biased results. First, we restricted the presence/absence matrix to bands whose observed frequencies were less than 1 - (3/*N*) where *N* is the mean number of sampled individuals per population (Lynch and Milligan, 1994). Second, in an initial analysis we visualized the molecular indices with the software 'Barrier'

(Manni et al., 2004) in order to detect patterns of molecular contrast that geographically coincide with borders of proposed post-glacial migration areas (Schönswetter et al., 2005). In such a case we restricted the analysis to populations within the same area avoiding transborder effects. Third, for the calculation of diversity and differentiation measures that are valid for interspecies comparisons, we matched the number of populations, individuals, and loci for each species through multiple random reductions of the parameters (100 subsamples) similar to the approach of Leberg (2002). Even though the information on the heterozygosity of populations was lacking, we assume that Hardy-Weinberg equilibrium was not violated. Pollination experiments in *G. reptans* and *C. thyrsoides* showed that both species are obligatory outbreeders with low seed set after self-pollination and no subsequent germination (Rusterholz et al., 1993; Ægisdóttir et al., 2006). *E. fleischeri* is known to be largely outbreeding but having the potential for selfing (Theurillat, 1979, Stöcklin, unpublished). For this species, repeated calculations with varying F_{is} from 0 to 1 at 0.25 step intervals increased AMOVA-derived Φ_{st}-values but maximum increase was less than 1 %. All statistical analyses(Aeschimann et al., 2005) were restricted to polymorphic bands and all computing was performed in 'R' (Ihaka and Gentleman, 1996) using the R-libraries 'ade4' (Thioulouse et al., 1997), 'vegan' (Dixon, 2003), 'smatr' (Warton et al., 2005) and self-written code.

Two commonly used indices of molecular diversity within populations were calculated: (1) Nei's expected heterozygosity H_e (Nei, 1978), and (2) the percentage of polymorphic bands (P_p). To quantify the variation of molecular diversity among populations, we calculated the coefficient of variation (CV) for H_e and P_p and compared species specific indices with univariate ANOVA and pairwise t-tests. For each species we assessed the correlation of H_e and P_p using Pearson correlation statistics. The relation between population sizes and molecular indices was calculated as a nonparametric Spearman's Rho (r_s) correlation. Moreover, the relation of altitude and molecular diversity was assessed as a linear regression (data only for *G. reptans* and *C. thyrsoides* available). Population differentiation, or among-population diversity, was calculated using AMOVA-derived fixation index Φ_{st} (Analysis of MOlecular VAriance, Excoffier et al., 1992). The species specific variance of Φ_{st}-values, obtained from multiple random reduction subsampling (see above), were then compared with a univariate ANOVA.

To test for isolation by distance (Slatkin, 1987), we applied Mantel test statistics correlating the genetic distance matrix (pairwise Φ_{st} values) and the geographic distance matrix (Euclidean square distances). Significance levels were obtained after performing 10100 and 10000 random permutations for the pairwise genetic distances (Φ_{st}) and the Mantel

test respectively. We used standardized major axis regression (SMA) to quantify the pattern of linear covariation (Rousset 1997) and compared species-specific regression slopes using one-sample tests with bootstrapping (n = 10000) over independent population pairs as implemented in 'smatr' (Warton et al., 2005).

Further, we calculated an UPGMA cluster analysis (Unweighted Pair Group Method with Arithmetic Mean) of pairwise Nei's unbiased genetic distances (Nei, 1978) to test for spatial separation, and displayed the results as dendrograms. Stable clusters were indicated (*) according to the 50 % majority rule (Lowe et al., 2004) after bootstrapping of 10000 replicates.

Results

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

Molecular diversity within populations

Mean genetic diversities, H_e, were similar in all species (*E. fleischer*: H_e = 0.19, *G. reptans*: H_e = 0.21; *C. thyrsoides*: H_e = 0.20) but significantly higher for *G. reptans* ($P < 0.05$) compared to the other two species. No difference was detected between *E. fleischeri* and *C. thyrsoides* ($P = 0.069$). The percentage of polymorphic loci, P_p, was significantly different

Table 2. Molecular diversity and differentiation indices of three alpine plant species. H_e: Nei's genetic diversity; P_P: percentage of polymorphic loci; $\boldsymbol{\Phi_{st}}$: AMOVA-derived fixation index $\boldsymbol{\Phi_{st}}$, standardized using multiple random reductions; IBD: Isolation by distance; $p\boldsymbol{\Phi_{st}}$: pairwise $\boldsymbol{\Phi_{st}}$; *pgeo*: pairwise geographic distance.

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

between all three species ($P < 0.05$) with *E. fleischeri* showing highest ($P_p = 74.8$), *G. reptans* intermediate ($P_p = 70.1$) and *C. thyrsoides* lowest ($P_p = 61.8$) levels of polymorphism. Summary statistics for species specific diversity indices are presented in Tab. 2; the population specific indices are listed in Appendix 1. In all three species H_e and P_p were positively correlated (*E. fleischeri*: *cor* = 0.74, $P < 0.001$; *G. reptans*: *cor* = 0.70, $P < 0.001$, *C. thyrsoides*: *cor* = 0.46, $P < 0.01$). In general, within-population measures of *G. reptans* based on 47 loci (this study) were similar or identical to the results based on 49 loci (Pluess and Stöcklin, 2004a). Furthermore, we detected no influence of population size on the molecular diversity of *G. reptans* (H_e: $r_s = -0.02$, $P = 0.95$; P_p: $r_s = -0.11$, $P = 0.64$) or *C. thyrsoides* (H_e: $r_s = 0.29$, $P = 0.22$; P_p: $r_s = 0.04$, $P = 0.88$). Population sizes for *E. fleischeri* were not estimated in the field and thus, could not be included. Molecular diversity was not related to altitude, except for a single significant increase of P_p with increasing altitude in *E. fleischeri* (*E. fleischeri*: H_e: $r^2 = 0.25$, $P = 0.1$; P_p: $r^2 = 0.19$, $P = 0.03$, *G. reptans*: H_e: $r^2 = 0.1$, $P = 0.1$; P_p: $r^2 = 0.01$, $P = 0.3$; *C. thyrsoides:* H_e: $r^2 = -0.04$, $P = 0.66$; P_p: $r^2 = -0.05$, $P = 0.86$).

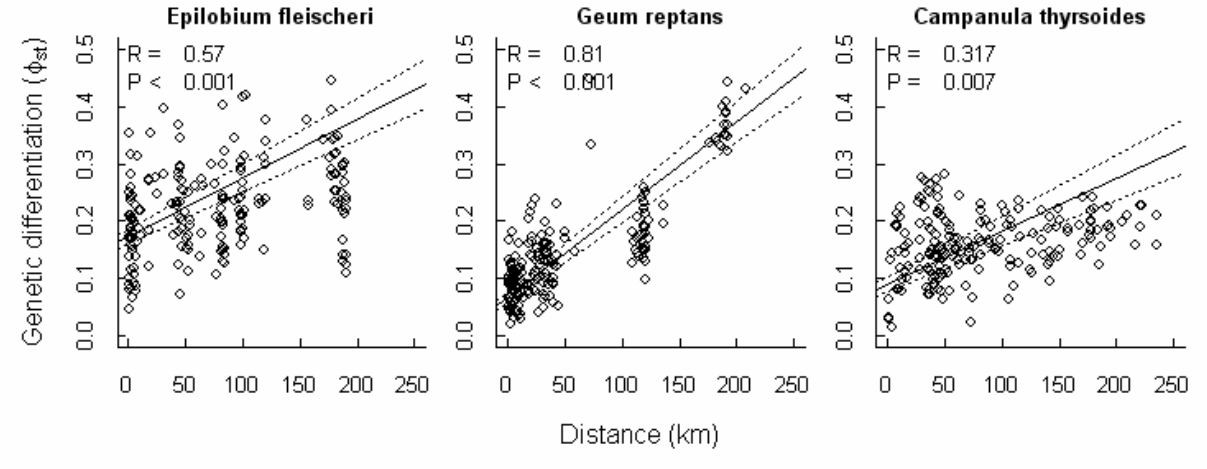

Fig. 2: Matrix correlation of genetic (pairwise Φ_{st} values) and geographic distances. Solid line: standardized major axis (SMA) regression; dotted lines: SMA 95 % confidence interval.

Spatial differentiation

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

For all three species we found a significant isolation by distance (IBD) pattern as calculated with Mantel test statistics (Fig. 2, Table 2; *E. fleischeri*: $R = 0.57$, $P < 0.001$; *G. reptans*: $R = 0.81$, $P < 0.001$; *C. thyrsoides*: $R = 0.32$, $P = 0.007$). The slope of the standardized major axis regression lines (SMA) was significantly steeper in *G. reptans* compared to the other two species (each $P < 0.001$), while no difference was found between *E. fleischeri* and *C. thyrsoides* ($P = 0.12$). The equations for the (SMA) including the slope and intercept specific standard errors are described as follows: *E. fleischeri*: y = 0.01729 (0.0062) + 1.025 10^{-03} (6.159 10^{-05}) x; *G. reptans*: y = 0.06563 (0.00512) + 1.536 10^{-03} (6.512 10^{-05}) x; *C. thyrsoides*: 0.08874 (0.00671) + 9.34 10^{-04} (6.502 10^{-05}) x, $R = 0.089$.

UPGMA cluster analysis (Fig.3) and application of Monmonier's algorithm (results not shown) for *E. fleischeri* and *G. reptans* revealed no geographic patterns of genetic

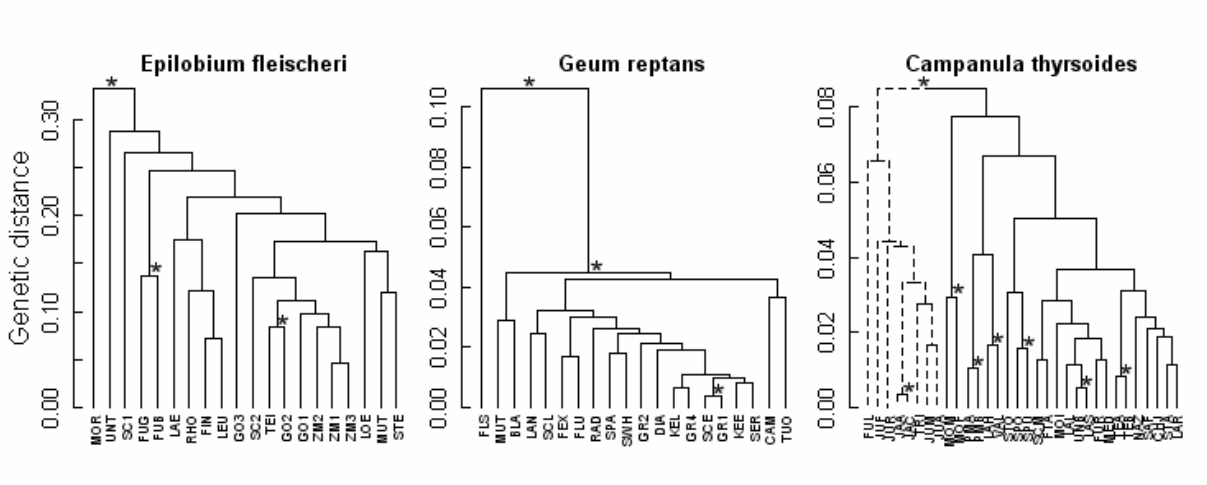

Fig. 3: Dendrograms of the UPGMA cluster analysis based on Nei's (1978) unbiased measure of genetic distance (* indicate bootstrap values larger than 50%, based on 10 000 permutations). Dashed clusters represent populations belonging to a different glacial refugia.

differentiation that coincide with the proposed areas of post-glacial migration in the western Alps (Schönswetter et al, 2005). In both species, all population pairs were significantly differentiated but stable dendrogram clusters were only present for a single geographically isolated population each (MOR and FLS, respectively). Even populations in close vicinity did not consistently group together. In *C. thyrsoides*, with both methods we detected a clear seperation of populations located in western Switzerland from those in central and eastern regions with the north-south running Aosta-Rhône-Valley as the geographic border. Within the two main UPGMA clusters, stable branches were mostly formed by population pairs separated by distances below 2 km. Nevertheless, two population pairs with distances of 49 and 73 km from each other formed stable ties (LAH, VAL, and UNB, LAS, respectively). All population pairs were significantly differentiated.

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

lines among the three regions were all significantly different ($P < 0.001$), steepest for western populations (n = 8), intermediate for the central/eastern ones (n = 24), and lowest for all populations (n = 32).

Discussion

Spatial isolation and genetic differentiation

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

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

provide indication that genetic diversity patterns in the alpine are not shaped by natural fragmentation alone.

Still, it is important to ask whether IBD is a characteristic feature of alpine plant populations, and whether IBD is more pronounced under alpine compared to lowland conditions. The few studies focussing on alpine plants find inconsistent IBD relationships and the significance of an IBD pattern to be dependent on population sub-grouping or, as discussed above, on geographic scale and post-glacial migration history. For example, in *Eryngium alpestris* no significant IBD was found at a geographic distance of 250 km (Gaudeul et al., 2000). In contrast, when subdivisions of 2 of these 14 populations were acknowledged the overall IBD was significantly positive. For the subdivided populations within individual valleys, a positive IBD was only found at distances up to 0.2 or 2 km. Such an effect of population subdivision was not found in any of our study species (data not shown). In a study on *Rumex nivalis*, IBD was only significant within a single large region of Switzerland (the same glacial refugia investigated in the present study), but the correlation was non-significant when populations from larger distances were included (Stehlik, 2002). Moreover, a significant IBD could be found in *Hypericum nummularium* for populations in the Alps, but IBD was lacking in the Pyrenees (Gaudeul, 2006). As for alpine species, no clear indication of the causes governing an IBD pattern or its magnitude is visible for lowland species of fragmented or continous populations. Presence or absence of an IBD is, again, explained by a multitude of potentially important factors, such as time since colonization (Jacquemyn et al., 2004), general rarity (Dittbrenner et al., 2005), breeding system (Irwin, 2001), dispersal potential (Coleman and Abbott, 2003), ocean currents (Bond et al., 2005) or maximum geographic distance (Hilfiker et al., 2004; Moyle, 2006). In the present study, we standardized a number of the above mentioned factors that potentially influence IBD and we will discuss the relative role of specific life-history traits further down. It can however be said, that in the current absence of standardized comparative studies or meta-analyses with a large number of species, there is no ample evidence that alpine species behave differently than lowland species or that natural fragmentation enhances IBD.

Spatial isolation and within-population diversity

Our three species have similar values of mean genetic diversity (H_e = 0.19 to 0.21) which are in concordance with other alpine species such as *Eryngium alpestris* (H_e = 0.20, Gaudeul et al., 2000) and *Trollius europeus* (H_e = 0.22, Despres et al., 2002) or the rather

wide range of H_e found in many other alpine or lowland species (see Till-Bottraud and Gaudeul, 2002; Nybom, 2004). The results for mean polymorphic loci are much more diverging but nevertheless high and show a considerable amount of variance (P_p = 62 - 75, Table 2). It has to be born in mind that measures of H_e are considered less sensitive to detect consequencs of isolation and population bottlenecks than alternatives such as allelic diversity (Amos and Balmford, 2001). Therefore, we would expect to find an effect of fragmentation and an effect of declining population sizes with measures of P_p, rather than H_e. Particularly in species with a short generation time low levels of P_p should be found (e.g. Young et al., 1996; Till-Bottraud and Gaudeul, 2002). Our data does show the lowest levels of P_p in the relatively short-lived *C. thyrsoides* as compared to the long-lived, clonal *E. fleischeri* or *G. reptans*. However, levels of P_p in *C. thyrsoides* are still high and we do not find a significant correlation between population size and P_p even though a number of populations are comprised of less than 100 reproductive individuals. This result suggests that even small populations of *C. thyrsoides* with only 45 flowering individuals are large enough to maintain high levels of genetic diversity and, as has been shown recently, do not need to suffer from inbreeding depression (Ægisdóttir et al., 2006). Genetic diversity was also not related to altitude so that the adaptive potential to buffer consequences of global warming is likely to be similar in all populations, irrespective of their altitudinal position. The single significant increase of P_p in *E. fleischeri* with increasing altitude shows a poor correlation and can be shown to be an artefact of primer selection. In this one case, the significance of the correlation was highly influenced by only five loci of a single primer. Simulations with the complete data set of 89 polymorphic loci of 10 primers showed not significant difference of P_p in relation to altitude. In general, we have no indication that natural fragmentation has led to lasting consequence on within-population diversity, given the high values of H_e and P_p in all three species and the absence of a population size effect.

Genetic differentiation and life-history traits

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

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

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

Unfortunately, up to date there is no study that attempted to elucidate the complex interaction of life-history traits on genetic diversity partitioning in a standardized geographic setting. In this context, it is important to note that for the above mentioned alpine species we did not use the 'global Φ_{st}-values' of each literature source but those values associated with 'genetic differentiation among populations within regions' in which case the size of a region

was similar to our study. This reduced the bias of geographic scale which may have been the reason behind a high 'mean RAPD Φ_{st}-value' of 27 % for outcrossing species as listed in a review by (Nybom, 2004). To conclude at this point, our three species have Φ_{st}-values between 14.8 % and 22.7 % which demonstrate a relatively restricted differentiation of populations and thus, we cannot confirm our initial hypothesis that natural fragmentation in the alpine environment has led to a particularly high population differentiation. The biology of a species appears to have a major influence on genetic diversity partitioning and largely masks an effect of spatial isolation.

Conclusion

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

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Appendix S1. Population descriptions of *Epilobium fleischeri*, *Geum reptans* and *Campanula thyrsoides*. Sampling locations, population abbreviation, co-ordinates, elevation, number of sampled plants per population (N), population size, H_e: Nei's expected heterozygosity, P_p: percentage of polymorphic loci. NA: not available. Calculations are based on 47 polymorphic loci.

Epilobium fleischeri

	Location	Population	Co-ordinates[a]	Elevation[b]	N	Pop. size	H_e	Pp (%)
1	Zermatt, Zmutt, VS	ZM1	617' 580/095' 125	2260	20	NA	0,20	82,69
2	Zermatt, Zmutt, VS	ZM2	618' 600/094' 555	2183	20	NA	0,18	71,15
3	Zermatt, Zmutt, VS	ZM3	618' 980/094' 760	2189	20	NA	0,21	86,54
4	Teifbach, VS	TEI	621' 620/094' 780	1930	20	NA	0,18	73,08
5	Furgg, VS	FUG	620' 000/092' 925	2435	20	NA	0,22	80,77
6	Furggbach, VS	FUB	621' 145/093' 675	2000	20	NA	0,20	69,23
7	Gornergletscher, VS	GO1	623' 180/092' 510	2100	20	NA	0,17	71,15
8	Gornergletscher, VS	GO2	623' 020/092' 870	2050	20	NA	0,22	78,85
9	Gornergletscher, VS	GO3	622' 130/093' 710	2020	20	NA	0,18	75
10	Findelgeltscher, VS	FIN	628' 880/095' 025	2470	20	NA	0,20	78,85
11	Leukerbad, VS	LEU	613' 600/137' 510	1700	20	NA	0,14	73,08
12	Lämmerenboden, VS	LAE	610' 650/138' 500	2310	20	NA	0,20	76,92
13	Loetschental, VS	LOE	635' 910/144' 375	2090	20	NA	0,20	67,31
14	Unteralptal, UR	UNT	691' 100/164' 650	1560	20	NA	0,15	65,38
15	Muttgletscher, VS	MUT	673' 700/158' 120	2060	20	NA	0,19	75
16	Rhonegletscher, VS	RHO	672' 050/158' 100	1790	20	NA	0,22	78,85
17	Steingletscher, BE	STE	675' 850/175' 350	1940	20	NA	0,21	76,92
18	Scaletta, GR	SC1	790' 200/176' 525	2100	20	NA	0,19	75
19	Scaletta, GR	SC2	790' 925/175' 900	2250	20	NA	0,21	80,77
20	Morteratsch, GR	MOR	791' 800/145' 500	2010	20	NA	0,13	59,62

Geum reptans

	Location	Population	Co-ordinates[a]	Elevation[b]	N	Pop. size	H_e	Pp (%)
1	Fluhseeli, BE	FLS	604' 700/139' 700	2070	17	1500	0,16	48,98
2	Muttgletscher, VS	MUT	674' 500/156' 600	2520	18	5000	0,22	63,27
3	Blauberg, UR	BLA	675' 030/157' 920	2580	17	7000	0,22	65,31
4	Val Fex, GR	FEX	781' 325/137' 730	2140	20	3500	0,24	81,63
5	Diavolezza, GR	DIA	794' 025/143' 500	2980	19	1000	0,24	71,43
6	Val da Cambrena, GR	CAM	797' 100/142' 300	2340	20	8000	0,22	65,31
7	Piz Languard, GR	LAN	793' 075/151' 450	3080	20	1500	0,22	73,47
8	Vadret da Porchabella, GR	KEE	787' 100/168' 020	2680	20	5000	0,22	69,39
9	Vadret da Porchabella, GR	KEL	785' 165/168' 460	2340	20	5000	0,22	79,59
10	Sertig, Gletschtälli, GR	SER	787' 450/173' 800	2460	20	5000	0,21	69,39
11	Scalettapass, GR	SPA	789' 935/174' 380	2600	20	1500	0,19	67,35
12	Scaletta, GR	SCE	791' 600/175' 430	2500	20	2000	0,22	73,47
13	Scaletta, GR	SCL	791' 750/175' 500	2330	20	8000	0,22	73,47
14	Vadret da Grialetsch, GR	GR4	792' 785/175' 850	2630	20	3000	0,23	81,63
15	Vadret da Grialetsch, GR	GR2	793' 220/175' 380	2660	20	9000	0,25	73,47
16	Vadret da Grialetsch, GR	GR1	793' 800/175' 300	2600	20	2000	0,22	79,59
17	Vadret da Rado¨nt, GR	RAD	792' 585/178' 485	2640	20	4000	0,23	79,59
18	Flüela Schwarzhorn, GR	SWH	791' 400/178' 750	2900	16	500	0,22	69,39
19	Flüelapass, GR	FLU	791' 700/180' 300	2420	19	4000	0,19	71,43
20	Vadret Tuoi, GR	TUO	806' 275/191' 300	2610	20	5000	0,21	69,39

Campanula thyrsoides

	Location	Population	Co-ordinates[a]	Elevation[b]	N	Pop. size	H_e	Pp (%)
1	Col du Marchairux, VD	JUM	508' 900/156' 400	1440	23	1000	0,17	53,19
2	Les Amburnez, VD	JUA	507' 480/155' 100	1340	23	1000	0,22	57,45
3	Pre du Rolle, VD	JUR	508' 983/155' 652	1377	23	150	0,16	57,45
4	Pres de Four, VD	JUF	498' 400/148' 450	1430	23	10000	0,21	65,96
5	Col du Jamon, VD	JAA	564' 830/145' 050	1630	23	100	0,18	59,57
6	Col du Jamon, VD	JAC	564' 589/144' 944	1670	23	80	0,20	63,83
7	Lac du Fully, VS	FUL	574' 000/113' 200	2100	23	500	0,21	57,45
8	Trient, Les Tseppes, VS	TRI	564' 350/099' 500	2020	23	50	0,24	65,96
9	Lac du Moiry, VS	MOI	609' 932/109' 638	2266	23	50000	0,21	57,45
10	Stockhorn, BE	STO	607' 737/171' 103	1980	23	100	0,22	68,09
11	Schynige Platte, BE	SPO	636' 225/167' 625	1990	23	600	0,22	68,09
12	Schynige Platte, BE	SPU	636' 600/167' 150	1890	23	500	0,20	57,45
13	Furka, UR/VS	FUR	674' 850/158' 825	2430	23	30000	0,22	68,09
14	Unterschächen, Butzlichöpf, UR	UNB	702' 500/193' 200	1900	23	500	0,20	55,32
15	Langwies, Listboden, GR	LAL	776' 750/191' 510	2000	23	300	0,18	59,57
16	Langwies, Strassberg, GR	LAS	775' 875/190' 550	1870	23	7000	0,21	76,6
17	Langwies, Holzbüel, GR	LAH	775' 010/188' 875	1700	23	50	0,19	53,19
18	Vals, Peil, GR	VAL	735' 375/160' 425	1850	23	100	0,20	70,21
19	Safiental, GR	SAF	742' 851/174' 289	1857	23	50	0,22	61,7
20	Medels, Parjurs, GR	MED	742' 800/157' 700	1870	23	50	0,22	61,7
21	Monstein, Mäschenboden, GR	MOM	779' 668/173' 708	1961	23	45	0,18	68,09
22	Monstein, Fanexmeder, GR	MOF	780' 750/174' 910	2220	23	250	0,19	59,57
23	Parsennmeder, GR	PMA	784' 030/191' 473	1995	23	5000	0,20	57,45
24	Parsennmeder, GR	PMB	784' 478/191' 548	1910	23	100	0,19	57,45
25	Churwalden, Joch, GR	CHJ	762' 300/185' 100	1890	23	150	0,21	59,57
26	St. Antönien, GR	STA	782' 203/201' 989	1943	23	250	0,22	61,7
27	Alp Laret, GR	LAR	784' 234/153' 944	2180	23	300	0,19	55,32
28	Albula Pass, Naz, GR	NAZ	778' 193/162' 751	1755	23	150	0,21	63,83
29	Schuol, La Motta, GR	SCM	816' 400/188' 400	2142	23	2000	0,19	57,45
30	Ftan, Prui, GR	FTA	812' 505/187' 750	2100	23	150	0,18	55,32
31	Tschlin, Alp Tea, GR	TEA	828' 250/198' 250	2200	23	150	0,20	61,7
32	Tschlin, Alp Tea, GR	TEB	827' 800/198' 000	2150	23	200	0,19	63,83

[a] Co-ordinates according to the Swiss topographical maps (Bundsamt für Landestopographie, Wabern, Switzerland).

b Elevation in meters above sea level

Chapter 7

Evolutionary demography of long-lived monocarpic perennials: a time-lagged Integral Projection Model

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Summary

1 The evolution of flowering strategies (when and at what size to flower) in monocarpic perennials is determined by balancing current reproduction with expected future reproduction, and these are largely determined by size-specific patterns of growth and survival. However, because of the difficulty in following long-lived individuals throughout their lives this theory has largely been tested using short-lived species (< 5 years).

2 Here, we tested this theory using the long-lived monocarpic perennial *Campanula thyrsoides* which can live up to 16 years. We used a novel approach that combined permanent plot and herb chronology data from a 3-year field study to parameterize integral projection models (IPMs).

3 In common with many other monocarpic species, the rosette leaves of *C. thyrsoides* wither over winter and so size cannot be measured in the year of flowering. We therefore extended the existing IPM framework to incorporate an additional time delay that arises because flowering demography must be predicted from rosette size in the year before flowering.

4 We found that all main demographic functions (growth, survival probability, flowering probability, and fecundity) were strongly size-dependent and there was a pronounced threshold size of flowering. There was good agreement between the predicted distribution of flowering ages obtained from the IPMs and that estimated in the field using herb chronology. Mostly, there was good agreement between the IPM predictions and the direct quantitative field measurements regarding the demographic parameters λ, R_0 and T. Overall, we therefore conclude that the model captures the main demographic features in the field.

5 Elasticity analysis indicated that changes in the survival and growth function had the largest effect ($\approx$80%) on λ and were considerably larger than in short-lived monocarps. We found only weak selection pressure operating on the observed flowering strategy which was close to the predicted evolutionary stable strategy (ESS).

6 To conclude, the extended IPM accurately described the demography of a long-lived monocarpic perennial using data collected over a relatively short period. We could show that the evolution of flowering strategies in short- and long-lived monocarps seem to follow the same general rules but with a longevity-related emphasis on survival assurance.

Key words: *Campanula thyrsoides*; elasticity analysis; evolutionary stable strategy; flowering threshold; size structured populations.

Introduction

Plants and animals often delay reproduction for several years and understanding the ecological and evolutionary processes that underlie this behaviour is a classic problem in evolutionary biology (Cole, 1954). The main benefits of early reproduction accrue through reductions in mortality and generation time (Cole, 1954; Charnov & Schaffer, 1973; Roff, 1992; Stearns, 1992). In general, reductions in mortality increase fitness, whereas reductions in generation time only increase fitness under certain circumstances, and may have no effect on fitness in a density regulated population (Mylius & Diekmann, 1995). The costs of early reproduction are reduced fecundity and/or quality of offspring (Bell, 1980; Roff, 1992; Stearns, 1992).

The study of reproductive delays in plants is complicated because plants vary continuously in size and there is enormous variation in growth between individuals. This means the standard models, which assume that growth is deterministic, do not perform well when applied to plants (Rees *et al.*, 1999; Rees *et al.*, 2000). Clearly a mathematical framework is required that allows individuals to vary continuously in size while at the same time also allows for a variation in growth rates. Integral projection models can naturally accommodate both these essential features allowing plant populations to be modelled in an elegant framework which is easily parameterized using standard demographic data. When combined with methods for calculating measures of population growth, i.e. the net reproductive rate (R_0) and finite rate of increase (λ), as well as ideas from evolutionary demography this approach provides a powerful set of tools for exploring reproductive decisions in biologically realistic models (Rees & Rose 2002; Childs *et al.* 2003, 2004; Rees *et al.* 2004, 2006; Rose *et al.* 2005).

The integral projection model was first introduced by Easterling *et al*. (2000) and subsequently extended by Ellner & Rees (2006) to include species with complex demography. For this general class of models they provided theoretical results for (a) stable population theory for density-independent models including sensitivity/elasticity analysis, (b) local stability analysis in density-dependent models (Ellner & Rees 2006). The models eliminate the need to divide data into discrete classes, without requiring any extra biological assumptions (Easterling *et al.* 2000). So far, the existing class of models assume that demographic rates are only influenced by current individual state, say size and/or age. In many species, however, the decision to flower is made many months (e.g. *Agave deserti*; Tissue & Nobel 1990) or years (*Frasera speciosa*; Taylor & Inouye 1985) before flowering occurs in the field. Additionally, flowering individuals of monocarpic perennials often have a

different morphology, e.g. withered or absent rosette leaves, such that size measurements in the year of flowering cannot be compared with the years prior to flowering (e.g. *Campanula sibirica*, *C. spicata*; Hegi 1975). In these latter cases, an additional time lag needs to be incorporated into the models in order to faithfully represent the demography of a study species.

The demography of monocarpic plants has been summarized by Metcalf *et al.* (2003) and for most species the basic demographic functions seem to follow a common pattern and are largely size-related: with increasing plant size the relative growth rate decreases, and fecundity as well as the probabilities of survival and flowering increases until after the first reproduction the plants die. The simple demography of monocarpic species allows testing of evolutionary ideas because the cost of reproduction is easily quantified and the timing of flowering a key determinant of Darwinian fitness. However, the theory on the evolution of flowering strategies of monocarpic plants is largely based on species with generation times of less than 5 years (Childs *et al*. 2003; Metcalf *et al.* 2003; Rees *et al.* 2006; Ellner & Rees 2006). This is largely a result of the practical difficulties inherent in studying long-lived individuals over their entire life-cycle. For many long-lived species then there is a need to develop and test new approaches that allow their demography to be understood using data that can be quickly collected. A promising method is the combination of size-structured modelling with age determination techniques for non-woody plants ('herb chronology'; Dietz & Ullmann 1998; Schweingruber & Poschlod 2005). In this way we can use the IPM to project the age-structure using the size-dependent demographic data and can compare it with the age-structure observed in the field.

Our objectives in this study are therefore 1) to present a theoretical and computational framework for a time-lagged integral projection model which allows demographic transitions to depend on an individual's state either in the current year or the year before, 2) to demonstrate that the novel combination of field methods (permanent plots and herb chronology) can be used to efficiently parameterize and validate IPMs with data from the long-lived monocarpic *Campanula thyrsoides* from a relatively short census period, 3) use the model to predict the age-structure observed in the field and determine the evolutionarily stable flowering strategy, and 4) to draw general conclusions about the evolution of the flowering strategies in long-lived vs. short-lived monocarpic plant species.

The estimation of model parameters, the graphics and the construction of the time-lagged integral projection model were performed with the software R (R Core Development Team 2006) and a script file is provided in Appendix S1.

Materials and Methods

INTEGRAL PROJECTION MODEL

The integral projection model describes how a continuously size-structured population changes in discrete time (Easterling *et al.* 2000; Ellner & Rees 2006). The state of the population is described by a distribution function $n(x,t)$, where $n(x,t)dx$ is the number of individuals with size in the range $[x,x+dx]$. The population dynamics are then

$$\begin{aligned} n(y,t+1) &= \int_L^U [P(y,x)+F(y,x)]n(x,t)dx \\ &= \int_L^U K(y,x)n(x,t)dx. \end{aligned} \qquad 1$$

Here $K(y,x)$, known as the kernel, describes all possible transitions from size x to size y, including births, and $[L,U]$ is the set of all possible sizes. The kernel is composed of two parts describing the production of size y offspring by size x parents, $F(y,x)$, and the movement of individuals from size x to size y, $P(y,x)$.

However, in many species a complication arises because either 1) rosette size cannot be measured in the year the plants flower or 2) the decision to flower is made the year before plant flower (Remark: most likely rosette size is a predictor of stored resources, though, we know nothing about how the decision to flower is made). As a consequence the probability of flowering and seed production in year t has to be predicted as a function of plant size in year t-1, and any seedlings produced then enter the population in year t+1. Therefore the time-lagged integral projection model becomes

$$n(y,t+1) = \int_L^U P(y,x)n(x,t)dx + \int_L^U F(y,x)n(x,t-1)dx \qquad 2$$

In order to apply the model we must specify the dependence of survival, growth and fecundity on size. This can be achieved by writing the fecundity function as

$$F(y,x) = p_e s(x) p_f(x) f_n(x) f_d(y) \qquad 3$$

where p_e is the probability of seedling establishment, $p_f(x)$ and $s(x)$ describe the probability that an individual of size x flowers or survives, respectively, $f_n(x)$ is the number of seeds it produces, and $f_d(y)$ is the probability density of seedling size y. The survival-growth function is given by

$$P(y,x) = s(x)[1-p_f(x)]g(y,x) \qquad 4$$

where $g(y,x)$ is the probability of an individual of size x growing to size y. The probability of flowering, $p_f(x)$, enters the survival-growth function because reproduction is fatal in monocarpic species.

The model can be solved numerically using the midpoint rule (Ellner & Rees 2006). To do this *mesh points* x_i have to be defined by dividing the interval $[L, U]$ evenly into m size classes and setting x_i at the midpoint of the ith class:

$$x_i = L + (i - 0.5)h, \quad i = 1, 2, \mathrm{L}\ , m \qquad 5$$

where $h = (U-L)/m$. The midpoint rule approximation to equation 2 is then

$$n(x_j, t+1) = h\sum_{i=1}^{m}[P(x_j, x_i)n(x_i, t) + F(x_j, x_i)n(x_i, t-1)], \qquad 6$$

which can be represented as a matrix multiplication

$$n(t+1) = \mathbf{K}n(t) \qquad 7$$

where $\mathbf{K}$ is the matrix of the form

$$\mathbf{K} = \begin{pmatrix} \mathbf{P} & \mathbf{F} \\ \mathbf{I} & \mathbf{0} \end{pmatrix}$$

where the $(i,j)^{\text{th}}$ entry of $\mathbf{F}$ is $hF(x_i,x_j)$, for $\mathbf{P}$ it is $hP(x_i,x_j)$, $\mathbf{I}$ is the $m \times m$ identity matrix, and $n(t) = (n(x_1,t), \mathrm{L}\ , n(x_m,t), n(x_1,t-1), \mathrm{L}\ , n(x_m,t-1))^T$. The number of mesh points generally depend on the study system and a range of mesh sizes should be explored to guarantee the accurate calculation of the demographic rates. For the *Campanula thyrsoides* model we used 100 mesh points for the size-dependent model and in the size × age model 50 age-classes.

Having constructed $\mathbf{K}$ it is then straightforward to calculate various statistics summarising population growth (λ, R_0 and generation time ($T = \ln(R_0)/\ln(\lambda)$); see Appendix S2 where we show that the general theory presented in Ellner & Rees (2006) can be applied to the time lagged model). The net reproductive rate R_0 is the long-term generation to generation population growth rate; so if g_0 is the total current population (generation 0), g_1 their total number of offspring counted at birth (generation 1), g_2 the total number of offspring they produce and so on, then

$$\lim_{k\to\infty} g_k / R_0^k = G \qquad 8$$

with the value of G depending on the initial population distribution. As in matrix models, R_0 and λ are related: $\lambda - 1$ and $R_0 - 1$ have the same sign (Ellner & Rees 2006). The additional time delay in reproduction affects when, but not how many, offspring are produced and so when calculating R_0, the time delay can be ignored leading to a slightly more efficient computation (Appendix S3). To obtain estimates of the uncertainty associated with the

various measures of population growth a bootstrap procedure can be used, see Appendix S4 for details.

For the calculation of the stable age distributions a size × age classified model can be constructed using the recipe given in Ellner & Rees (2006; Appendix A). The model is iterated until it converged on a stable state distribution. Note that this is slightly inefficient as the distribution of offspring sizes is usually independent of parental size and so the size × age structured model can be collapsed to a Leslie matrix; see Childs *et al.* 2003 Appendix A for details.

The matrix **K** is useful for calculating various measures of population growth; however, it is inappropriate for sensitivity and elasticity analyses because the identity matrix, **I**, in the lower left hand corner is simply a computational device and not a biological transition. To get around this problem direct perturbation of **P** and **F** can be used to calculate the sensitivities and elasticities, see Appendix A of Ellner & Rees (2006) for details. If $e_{\mathbf{F}}(y,x)$ and $e_{\mathbf{P}}(y,x)$ are the fecundity and growth-survival elasticities respectively, then because the **F** transitions take 2 year (they are transitions from year *t-1* to *t+1*) the fecundity elasticities need to multiplied by 2 in order for the elasticities to integrate to 1, specifically

$$2\iint e_{\mathbf{F}}(y,x)dydx + \iint e_{\mathbf{P}}(y,x)dydx = 1 \qquad 9$$

(see Appendix S5 for a more detailed discussion of this point).

In order to predict how the probability of flowering varies with plant size, the flowering strategy, we used the evolutionarily stable strategy (ESS) approach. The flowering strategy is characterized by a logistic regression with intercept β_0 and size slope β_s. When both of these parameters are allowed to evolve the predicted strategy is a step function; all plants should flower with probability 0 or 1 depending on whether their size is below or above a threshold (Rees & Rose 2002; Childs *et al.* 2003). However, many monocarps show a more gradual increase in the probability of flowering with size, suggesting a constraint or that the decision to flower is made sometime between censuses or there is genetic variation in the flowering threshold (Childs *et al.* 2003; Metcalf *et al.* 2003). We therefore constrain the ESS to be a gradual change in the probability of flowering with plant size by fixing the size slope, β_s, at its estimated value and allowing the intercept, β_0, to evolve. For systems in which density dependence acts primarily on the probability of establishment, e.g. *Campanula thyrsoides* (see below), the ESS can be characterized by maximising R_0 (Mylius & Diekmann 1995; Ellner & Rees 2006).

BIOLOGY OF THE SPECIES

A comprehensive description of *Campanula thyrsoides* L. (Campanulaceae) has been published by Kuss *et al.* (2007) and the following species information refer to this publication. *C. thyrsoides* is native to the European Alps and the adjacent mountain ranges to the East (Dinarids and Balcans) and North-West (Jura), and it is usually found from treeline ecotone into the alpine belt (1600 to 2500 m a.s.l.). The plant is rare but locally abundant with average populations consisting of a few hundred to several thousand individuals. Typical habitats are species-rich grasslands and screes on limestone or carbonate-bearing schists. Traditionally, *C. thyrsoides* has been considered to be a biennial species but plants can live up to 16 years growing significantly older with increasing altitude. The species shows two-phased rosette growth each year: a spring rosette which reaches maximum size at the end of the growing season, and a distinct summer rosette with smaller leaves that is initiated within the spring rosette few weeks prior to peak season conditions. Rosette leaves generally wither over winter. *C. thyrsoides* is usually unbranched but in less than 10% of all plants two or rarely more sister rosettes are formed either due to herbivory or other damage to the meristem. The sister rosettes are autonomous regarding size-related flowering thresholds but will die once flowering has been initiated in any sister rosette. *C. thyrsoides* forms a large tap root which can grow up to 1 m long, and flowering plants display a dense spike of around 100 yellow flowers that are pollinated by bumble bees and other hymenoptera. Individuals only reproduce by seed and can produce between 15,000 and 50,000 viable seeds. Seed dispersal is restricted to the close vicinity of the mother plant and there appears to be little long-distance wind dispersal. All seeds germinate after snowmelt the following spring and there is no evidence of a persistent seed bank.

DATA COLLECTION

Population data on *Campanula thyrsoides* were used from two sites in the Swiss Alps collected from July 2003 until August 2005. One site was at Schynige Platte (SP), Canton Berne (N 46°40’ E 7°54’, 1990 m, SW-exp., 30° slope), and the other at Furka Pass (FU), Canton Valais (N 46°34’ E 8°25’, 2430 m, S-exp., 40° slope). At both sites, individuals of *C. thyrsoides* were growing at low densities within alpine meadows. Twenty-one (SP) and thirty-five (FU) 1×1-m quadrats were distributed at random within each population, with the aim of having at least 300 plants per site. Within each quadrat, the position, number of leaves, length

and width of longest leaf (in mm) of each non-flowering plant were recorded (SP: end of July; FU: mid-August). For flowering individuals, we estimates seed production by counting the number of capsules and multiplied this by average seed per capsule (estimated from 5 capsules of 10 individuals per site). To test viability, a common garden experiment was established at FU and seeds from both sites were sown into soil collected in the field. Germination success was recorded after snowmelt in the following year. Viability of seeds and average seed production per capsule were combined to estimate the number of viable seeds that potentially could establish in a given plot. The total data set includes 515 individuals with 1371 observations from SP and 416 individuals with 998 observations from FU. For each year and site the annual population growth rates could then be calculated from the life-table entries: $\lambda = n(t+1)/n(t)$, where n is the number of individuals.

In the final season, 2005, plants from two plots at each site were harvested to measure above-ground biomass in addition to morphometric data. Stained cross sections of root collars were used to age each individual (SP: 122 inds.; FU: 132 inds.) applying standard herb chronology techniques (von Arx & Dietz 2006). Thin sections were photographed through the phototube of a dissecting microscope (Wild M3Z, Heerbrugg, Switzerland) using a digital camera (Nikon CoolPix 990, Nikon, Tokyo, Japan). In 90% of all plants, minimum and maximum age estimates differed by no more than one year. When different counts were obtained from the same individual, the mean of minimum and maximum age was taken (rounded up if the mean was not an integer) and used as an estimate of plant age. Mean generation time, T, for each population could then be calculated as the mean flowering age + one year. With the population growth rate, λ, and T known, it was straightforward to estimate the net reproductive rate, $R_0 = \lambda^T$ (Silvertown & Charlesworth 2005). It is important to note that the results from the herb-chronology investigation were compared to the IPM predictions and not used in the construction of the IPM.

PARAMETER ESTIMATION

As our measure of plant size we used the log-transformed product of number of leaves and length of longest leaf per vegetative rosette; this size parameter was highly correlated with the log-transformed above-ground dry weight ($r = 0.91$, $P < 0.001$, $y = 0.60 + 7.80\ x$) .The statistical models were then fitted to the combined data from both field sites, including site effects when they were significant. The fitted models are given in Table 1. Annual changes in

Table 1. Statistical models and parameter estimates describing demographic processes of *Campanula thyrsoides*. The models are functions of log rosette size, x, which is the product of the length of the longest leaf and the number of leaves (for details see text). The predicted values are the conditional mean, $\hat{y}$, and variance, σ_g^2, of log size next year given the current size; s: survival probability; p_f: flowering probability; f_n: fecundity; p_e: establishment rate. Figures in brackets are standard errors. Sites: Furka Pass (FU), Schynige Platte (SP)

Demographic process	**Site**	**Model**
Growth	FU:	$\hat{y} = 1.18(0.08) + 0.88(0.01)x$
	SP:	$\hat{y} = 1.07(0.09) + 0.88(0.01)x$
		Variance about the growth curve:
	FU:	$\sigma_g^2 = 4.60\exp(-0.49\hat{y})$
	SP:	$\sigma_g^2 = 4.60\exp(-0.42\hat{y})$
		$n = 1092$, $P<0.0001$
Survival probability	FU:	$\text{Logit}(s) = -0.48(0.57) + 0.63(0.12)x$
	SP:	$\text{Logit}(s) = 3.6780(1.12) - 0.11(0.18)x$
		$n = 1226$, $P<0.0001$
Flowering probability	FU:	$\text{Logit}(p_f) = -47.19(9.35) + 5.91(1.20)x$
	SP:	$\text{Logit}(p_f) = -22.04(3.37) + 2.74(0.44)x$
Fecundity (seeds per flowering plant)	FU:	$f_n = \exp(1.61(0.92) + 1.01(0.12)x)$
	SP:	$f_n = \exp(1.21(0.97) + 1.01(0.12)x)$
		$n = 53$, $r_s = 0.49$, $P<0.0001$
Probability of seedling establishment	FU:	$p_e = 0.00016$
	SP:	$p_e = 0.00078$
Distribution of seedling size	FU:	Gaussian $\mu = 3.33(0.03)$
	SP:	Gaussian $\mu = 4.00(0.08)$
		$\sigma^2 = 0.58$, $n = 671$, $P<0.0001$

plant size (growth) were size (P<0.0001) and site (P<0.0002) dependent, and were fitted by a linear model with size and site dependent variance (P<0.0001; see Fig. 1a). These models were fitted using generalized least squares and the significance levels were derived from likelihood ratio tests. The probabilities of flowering, $p_f(x)$, and of survival, $s(x)$, were each modelled using logistic regressions. In both cases the interaction between size and site were significant ($p_f(x)$: $\chi_1^2 = 8.46$, P<0.004, Fig. 1b; $s(x)$: $\chi_1^2 = 12.36$, P<0.0004, Fig. 1c) suggesting models with separate intercept and size slopes for each site. Seed production

(fecundity, f_n (x)) was modelled using linear regression and was size ($F_{1,50}$ = 62.0, P<0.0001) and site-dependent ($F_{1,50}$ = 7.15, P<0.01), suggesting a model with site-specific intercept and a common slope (Fig. 1d). We have no information on the distribution of seedling sizes from plants of different sizes and so we assumed seedling size to be independent of adult size. The distribution of seedling sizes in the field was site-dependent ($F_{1,669}$ = 65.92, P<0.0001) and modelled as a Gaussian distribution with site-specific mean and common variance. The probability of seed establishment was estimated by dividing the total number of seedlings recorded by the total seed production the previous year at each site.

Despite seed production per quadrat varying from 0 to 68,000 seeds the maximum number of seedlings recorded in a quadrat the following year was only 25. At both sites there was no relationship between per quadrat seed production and subsequent recruitment (FU: r_s = 0.09, P > 0.4, n = 70; SP: r_s = 0.17, P > 0.2, n = 42). At Furka Pass, plant densities per 1×1 m plot ranged from 2 to 70 individuals with a mean of 18.1 and at Schynige Platte from 1 to 56 individuals with a mean of 13.3. Our density-dependent model therefore assumes that population growth is limited by microsite availability. To evaluated the influence of the establishment rate, p_e, on λ, R_0 and T we iterated the model with varying p_e from 0.00001 to 0.001 while keeping all other IPM parameters constant.

Results

DESCRIPTIVE PROPERTIES OF THE MODEL

The descriptive properties of the parameterized model can be assessed by calculating the main demographic rates (λ, R_0 and T) as well as the stable size- and stable age-distribution numerically and comparing the estimates with the results obtained from life-tables and the age and size distribution in the field.

For each study site, the IPM-predicted population growth rates, λ, were within the 95% confidence interval of the corresponding across-year averages based on life-table calculations (Tables 2 and 3). The estimated site differences using the bootstrap approach outlined in Appendix S4 showed that λ and R_0 significantly differ between the FU and SP populations while generation time, T, did not (Table 2). For the FU population we found excellent agreement between the model prediction and direct measures. Here, both methods predict a moderate population increase (λ: 1.05-1.08). The agreement between the methods was less pronounced for the SP population, with the life-table calculations giving λ = 1.08 Figure 1

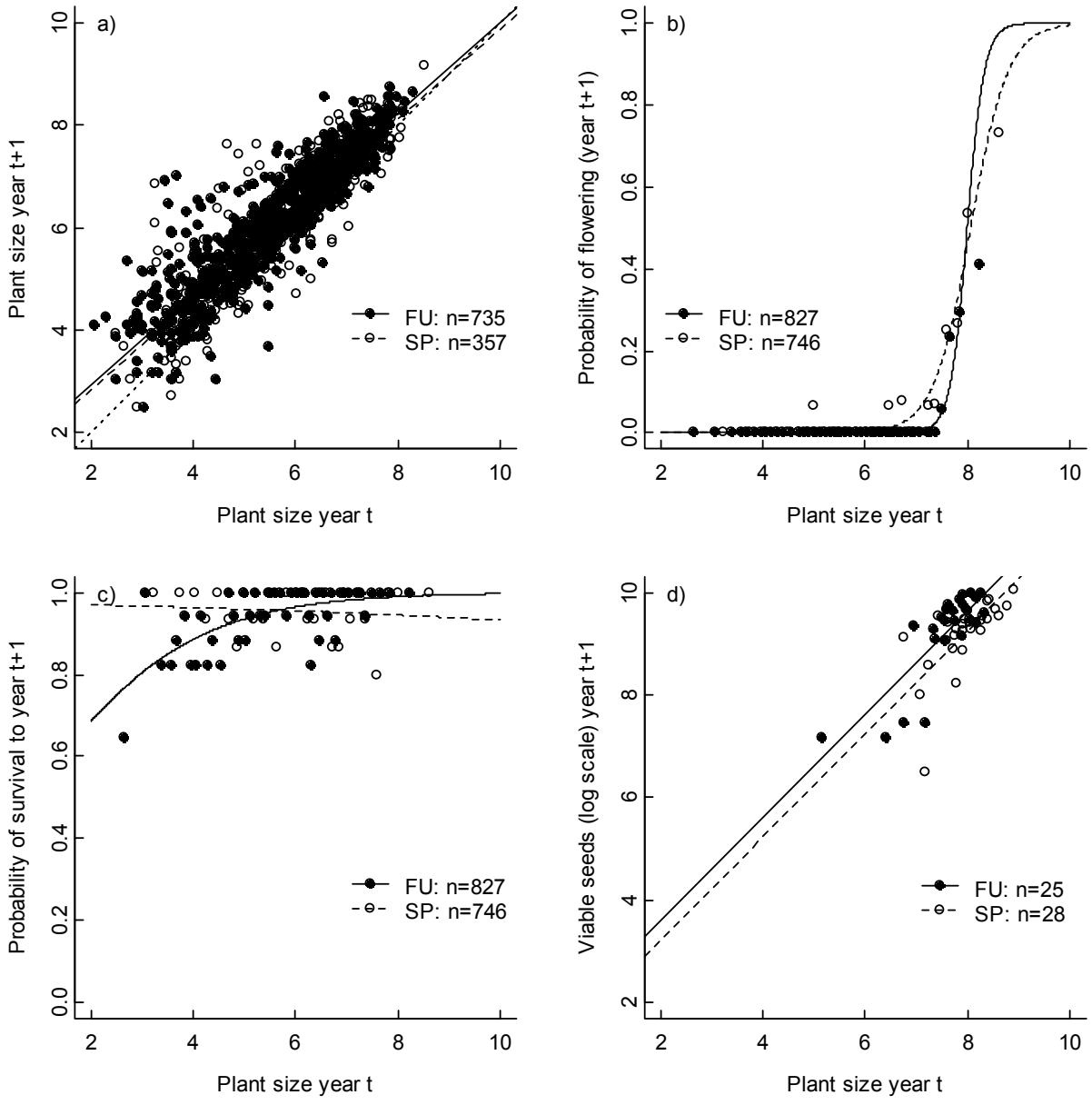

Figure 1. Demographic functions for *Campanula thyrsoides*: a) growth relationship for plant size in successive years, for reference the 1:1 line has been added (lower dotted line), b) probability of flowering relationship (Note: for plotting the relationship the data have been binned using 50 equal segments between plant sizes 2 and 10); however, all statistical analysis was performed on the binary data), c) probability of survival relationship (note: again for plotting the data have been binned), and d) fecundity relationship.Plant size represents rosette size: log (number of leaves * length of longest leaf). Sites: Furka Pass (FU), Schynige Platte (SP).

Table 2. Population growth rate, λ, net reproductive rate, R_0, and generation time, T, for *Campanula thyrsoides* extracted from the site-specific integral projection models. The figures in brackets are 95% confidence intervals based on 5,000 bootstrapped samples, see Materials and Methods as well as Appendix S4 for details.

Site \ Parameter	λ	R_0	T
Furka Pass	1.05	1.67	10.82
	(0.96, 1.12)	(0.64, 3.19)	(10.18, 11.77)
Schynige Platte	1.17	4.97	10.37
	(1.07, 1.30)	(2.19, 13.14)	(9.19, 11.70)
Site differences	-0.15	-4.44	0.58
	(-0.29, -0.02)	(-11.79, -0.35)	(-0.90, 1.94)

Table 3. Population growth rate, λ, net reproductive rate, R_0, and generation time, T, for *Campanula thyrsoides* extracted from the site-specific life-tables entries in combination with the herb chronology investigation. Values in square brackets are one year transitions, i.e. [2003/2004, 2004/2005]; see Materials and Methods for details.

Site \ Parameter	λ	R_0	T
Furka Pass	1.08	2.09	9.60
	[1.03, 1.14]	[1.33, 3.52]	SE = 0.66, n = 22
Schynige Platte	1.08	2.01	9.10
	[1.15, 1.01]	[3.57, 1.09]	SE = 0.41, n = 27

whereas the IPM predicted λ = 1.17, with a lower 95 % confidence interval of 1.07. Similarly, the net reproductive rate, R_0, did not differ significantly between the predicted model values and field observations (Tables 2 and 3). Again, we found better agreement between the results for the FU population than for the SP population. The mean generation time, T, estimated from the IPMs was approximately 10-11 years (Table 2), while T calculated with the herb

chronology data approximated to 9-10 years (Table 3). Hence, overall, there was good agreement between modelled and measured results.

Using herb chronology data we could look at the age structure of all plants and flowering individuals at each of the sites (Fig. 2). Superimposed on these distributions are the predicted stable age-distributions derived from the site-specific IPMs. At the Furka Pass the IPMs provided remarkable good description of the age structure of all plants and flowering ones. In contrast, at the Schynige Platte where the population is predicted to increase rapidly there were substantial differences with too few young individuals in the total population. Looking at flowering plants, however, the IPM provided a good description of the observed age distribution. Further, with the morphometric data from the permanent plots we could look at how well the predicted stable size structures from the IPM compare with that observed in the field (Fig. 3). At both sites the model predictions were fairly accurate and particularly good when just looking at flowering plants.

EFFECT OF SEEDLING ESTABLISHMENT RATES

The effect of varying the probability of seedling establishment, p_e, on λ, R_0 and T are shown in Fig. 4). At equal p_e, the FU population had a considerably higher value for λ and R_0 compared to the SP population, demonstrating that the differences in population growth observed between sites were not simply due to differences in the probability of seedling establishment. In contrast, the generation time at equal p_e was always higher for the SP than for the Furka population. These simulations indicate that populations can persist at extremely low establishment rates and population growth can increase dramatically by increasing microsite availability.

ELASTICITY ANALYSIS

We used elasticity analysis to partition the contributions of different sizes and types of life-history transitions to λ. Partitioning the elasticities into survival-growth and reproduction components of the kernel, we found that the survival-growth transitions were the critical determinant of λ. The summed survival-growth elasticities amounted to 0.81 and 0.78 at the FU and SP sites, respectively. The elasticities could be partitioned into contributions from plants of different sizes (Fig. 5). The contribution of survival- growth to λ was dominated by transitions into the larger size range where reproduction occurs (Figs 5a and 5c). For the

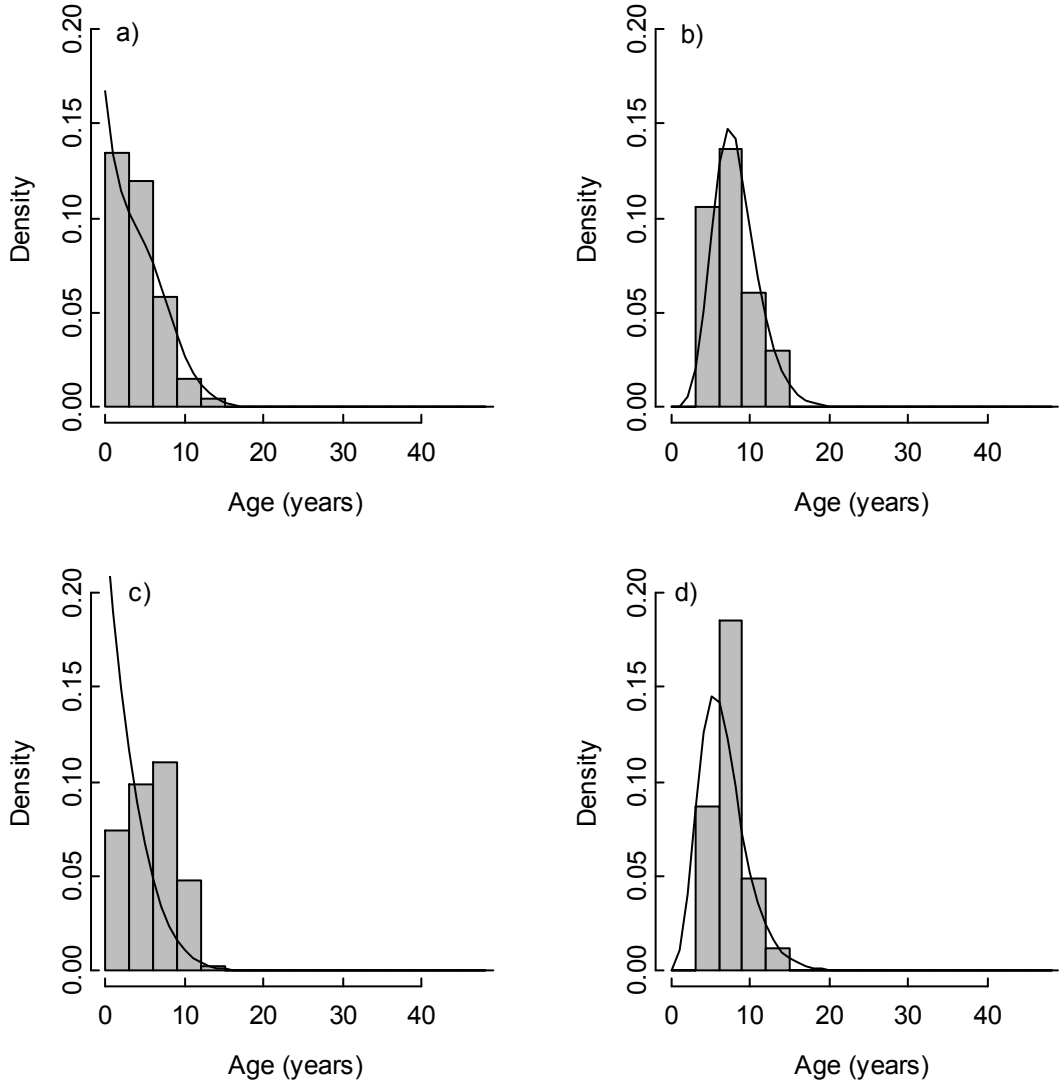

Figure 2. Age distributions of *Campanula thyrsoides* for the Furka Pass (FU: top row) and Schynige Platte population (SP: bottom row) sites, for all plants (a and c) and for flowering plants only (b and d). The solid bars are the data, the lines the predicted stable age distributions from the site-specific IPMs.

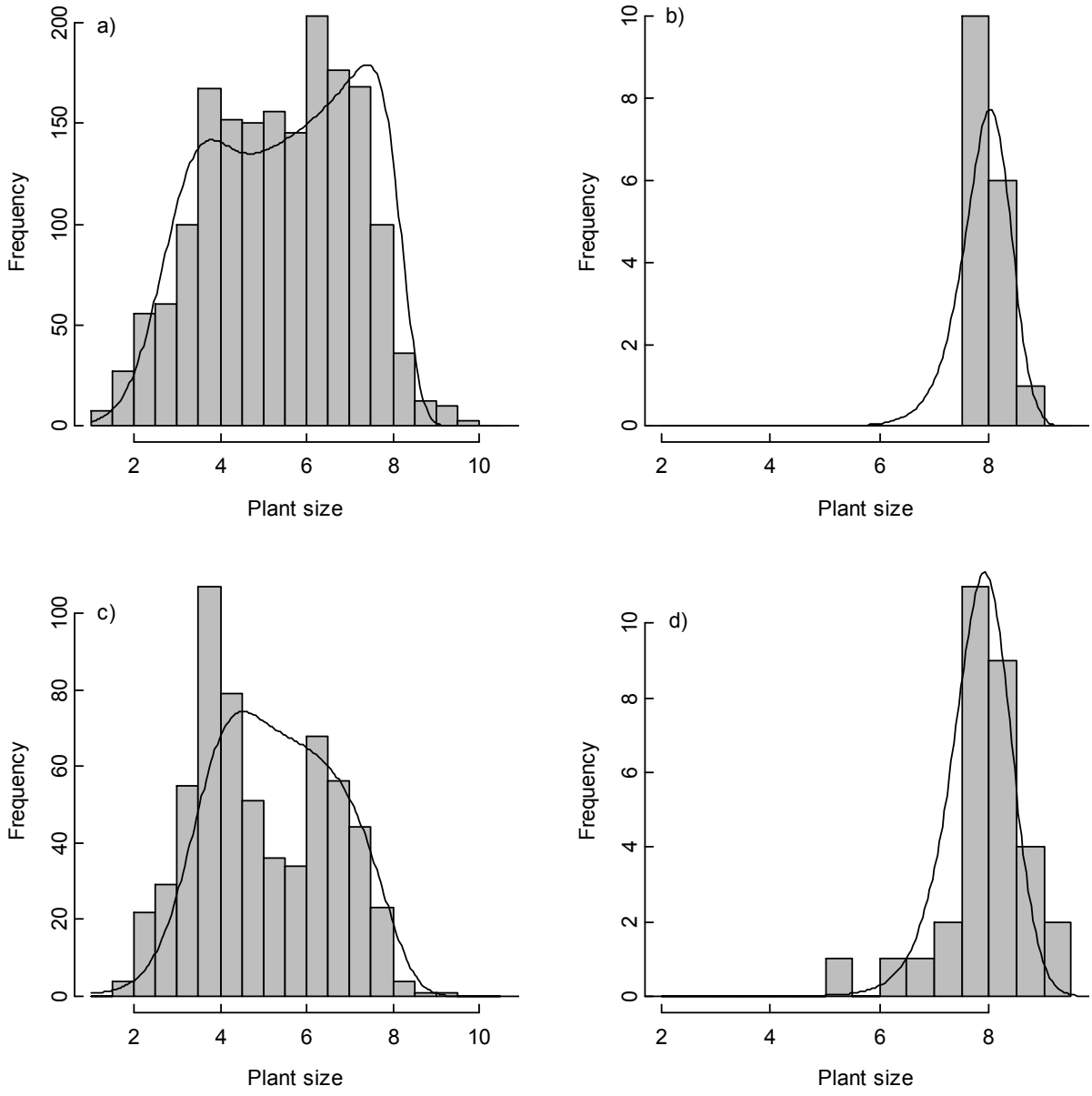

Figure 3. Size distributions of *Campanula thyrsoides* for the Furka Pass (FU: top row) and Schynige Platte population (SP: bottom row), for all plants (a and c) and for flowering plants only (b and d). The solid bars are the data, the lines the predicted stable age distributions from the site-specific IPMs. For details on size measurements see Material and Methods.

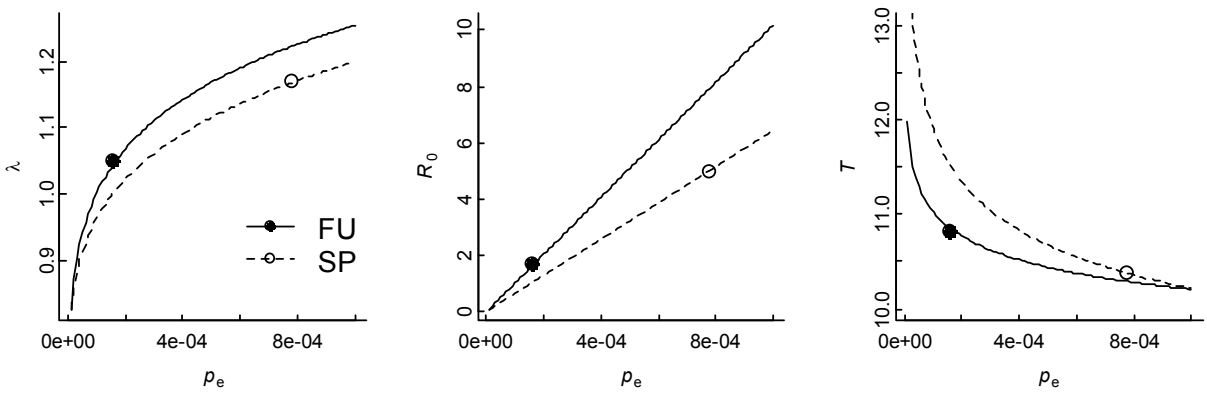

Figure 4. Predicted effect of changing the establishment rate p_e on population growth rate, λ, net reproductive rate, R_0, and generation time, T, for *Campanula thyrsoides*. Dots show observed values. Lines represent simulated results from the p_e interval 0.00001 to 0.001 (solid line: Furka Pass population (FU), dashed: Schynige Platte population (SP)

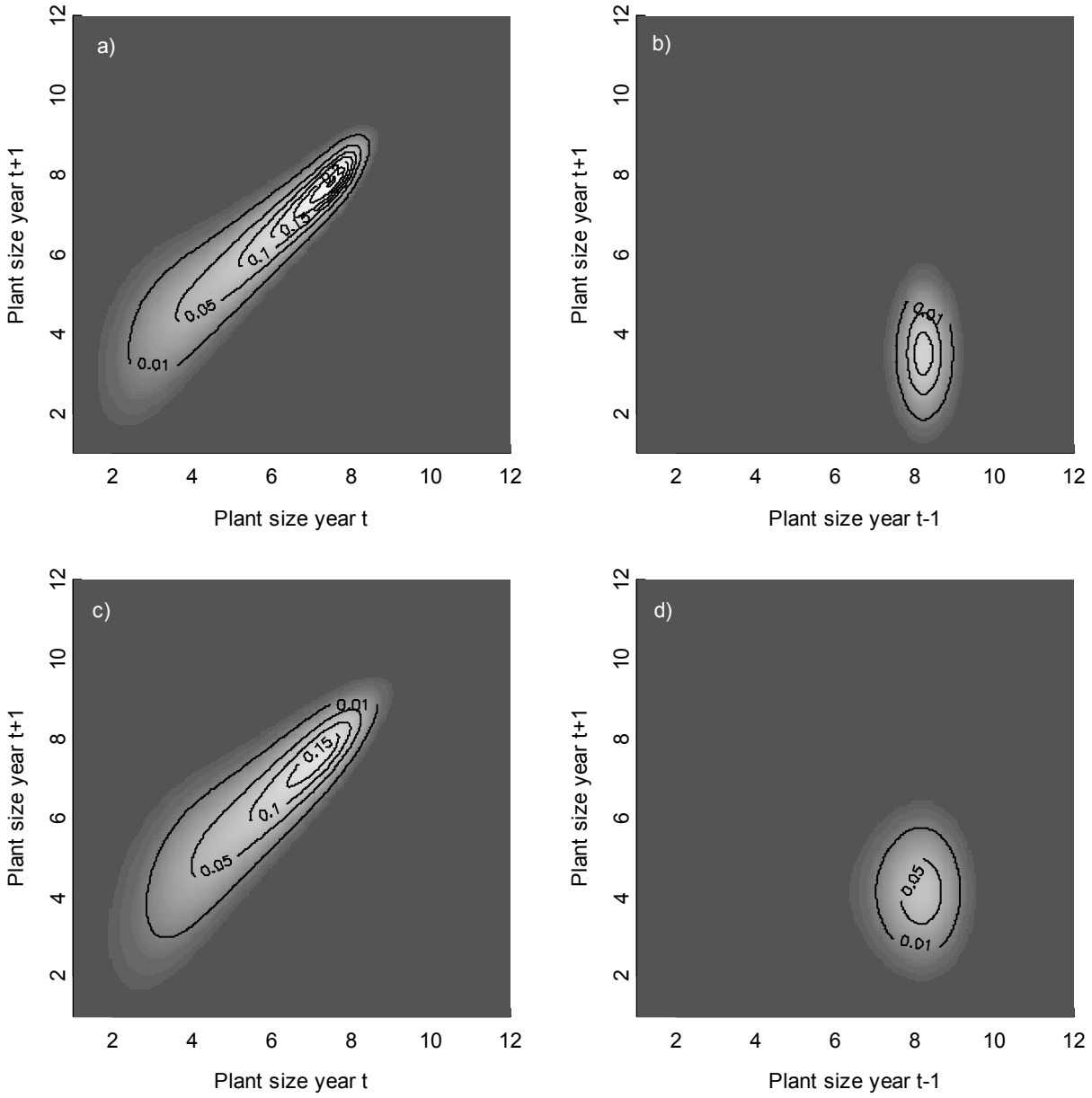

Figure 5. Survival-growth and fecundity elasticities for *Campanula thyrsoides* for the Furka Pass (FU: top row) and Schynige Platte population (SP: bottom row); survival-growth (a and c) and fecundity (b and d). Lighter colors represent areas of greater elasticity; contour lines mark elasticities of 0.01, 0.05, 0.1, 0.15, 0.2 and 0.25.

reproduction component of the kernel (Fig. 5) the contributions of different size transitions were dominated by movement of individuals from large sizes to recruits which is a consequence of larger individuals having higher probabilities of flowering and producing more seeds (Figs 5b and 5d).

EVOLUTIONARY ANALYSIS

As density dependence acts primarily on seedling establishment, the site-specific ESS flowering strategies maximise R_0 (Mylius & Diekmann 1995; Ellner & Rees 2006), and so the relationship between R_0 and the flowering intercept, β_0, defines an adaptive landscape (Figs 6a and 6c). At both sites, the ESS was not significantly different from the estimated value, although both site-specific ESSs were smaller and so plants following the estimated ESS would flower at slightly larger sizes than real plants. An alternative approach to testing whether the observed flowering strategies are ESSs is to plot the relationship between λ and β_0 (Figs 6b and 6d; de Jong and Klinkhamer 2005), using the density independent IPM (i.e. using the estimated probability of seedling establishment, p_e). In the approach used here, we simply ask if there are alternative flowering strategies that can invade, and do not attempt to predict the ESS flowering strategy. At Furka Pass the strategy maximising λ was similar to that which maximised R_0, which was to be expected at $\lambda \approx 1$, suggesting that the observed flowering strategy was an ESS. At Schynige Platte, however, the population was predicted to increase rapidly ($\lambda = 1.17$) which, as expected, selects for smaller sizes at flowering because this reduces generation time (Fig. 6d). In this case the observed strategy was extremely close to that which maximised λ.

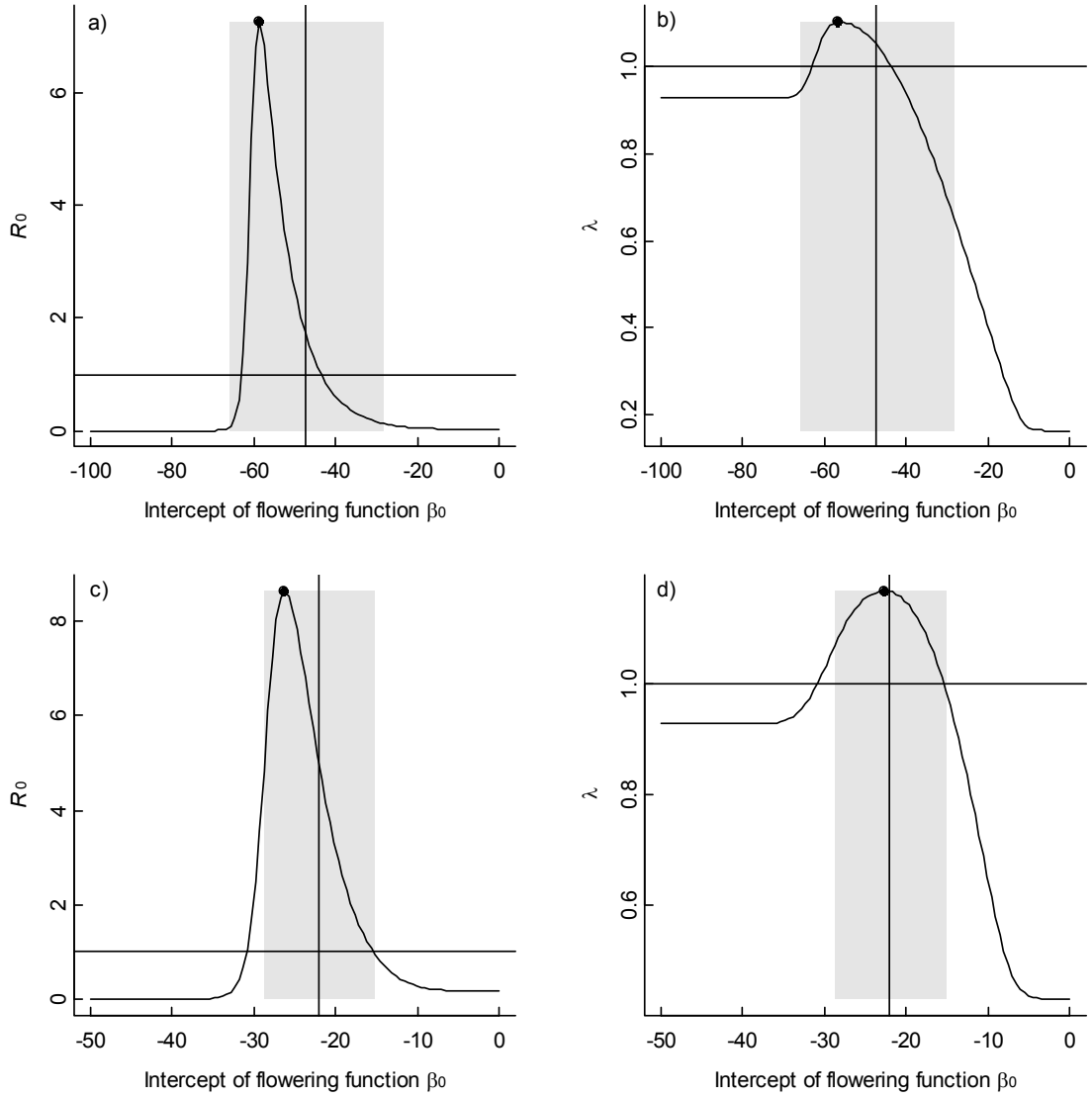

Figure 6. Predicted effects of changing the intercept of the flowering function β_0, on population growth rate, λ, and net reproductive rate, R_0, for *Campanula thyrsoides*: a) and b) Furka Pass site (FU), c) and d) Schynige Platte site (SP). Vertical lines represents observed β_0, horizontal lines the population equilibrium. The lines show the estimated values of λ and R_0 with varying β_0. Solid dots mark the ESS. The grey polygon describes the 2 SE uncertainty around the observed β_0.

Discussion

FIELD DATA AND TIME-LAGGED INTEGRAL PROJECTION MODELS

In this article, we have shown how to extend the IPM framework so that demographic transitions can depend on an individual's current or previous state. The resulting model has all the properties of the standard IPM (Ellner & Rees 2006) and is straightforward to parameterize. We then used the extended model to explore the demography of a long-lived monocarpic perennial in which plant size cannot readily be measured in the year of flowering. The estimated demographic parameters, i.e. λ, R_0, and T, as well as the stable age-distributions and stable size-distributions were largely in good agreement with the results obtained directly from field observations.

The incorporation of a time-lag extends the existing class of IPMs, allowing a wide range of life cycles to be explored. Time lags in this system simply change when offspring are produced and not how many are made, and so have no effect on R_0 (Appendix S3), and hence on the ESS flowering strategy. In contrast ignoring additional time-delay results in λ being over-estimated with a degree of error that depends on how rapidly the population is increasing, for FU the percentage error is $\approx 0.5\%$, whereas at SP it's $\approx 2\%$.

The time-lagged IPMs were successfully parameterized with data from a comparatively short census period of 3 years. Therefore, the novel combination of field methods, i.e. permanent plots and herb chronology, provide an elegant way parameterizing models and validating them for long-lived species, while bypassing the need to follow individuals over their entire life-cylce. There are, however, obvious limits to this approach, for example the short census period limits the temporal resolution of annual variability of demographic functions. This is particularly important for species showing sporadic seasonal synchrony (Janzen 1976; Taylor & Inouye 1985) or mast seeding behaviour (Taylor & Qin 1988; Rees *et al.* 2002). There are also potential problems with the detection of annual rings which is dependent on the study species. A number of biological constraints exist that can impede proper dating of root ages such as root centre decay, structural ring variability around the stem, alternating xylem-phloem structures, rhythmic bark structures as well as general climatic restrictions on annual ring formation (Schweingruber & Poschlod 2005).

COMMON PATTERNS OF SHORT- AND LONG-LIVED MONOCARPIC PERENNIALS

In many monocarpic perennials the four basic demographic functions (i.e. growth, probability of survival, probability of flowering, and reproductive output) have been shown to be strongly size-dependent (Metcalf *et al.* 2003). *C. thyrsoides* follows the same pattern. The relative growth rate was a decreasing function of size and we found a pronounced threshold size for flowering (Figs 1a and 1b). Flowering thresholds are common in monocarpic plants (Tissue & Nobel 1990; Wessenlingh *et al.* 1995; de Jong *et al.* 1998; Rose *et al.* 2002) and may represent the accumulation of a minimum amount of stored reserves required for flowering (Young & Augspurger 1990).

We observed a site-dependent variability in the probability of flowering, however at both site the size at which plants have a 50% chance of flowering was almost identical (Fig. 1b). The substantial variance about the growth curves was most probably the result of spatial heterogeneity in microsite-related growth conditions and/or the interannual variation of the length of the vegetation period since both factors are characteristic for alpine habitats (Körner 2003). This variance about the growth curve has also important genetic consequences because flowering within a cohort is spread over several years (Figs 3b and 3d) resulting in the probability of mating among close relatives and consequent inbreeding-depression being considerably reduced.

The probability of survival, $s(x)$, commonly increases with size in short-lived monocarpic plants (Metcalf *et al.* 2003), often with a considerable yearly variability (e.g. Rees *et al.* 1999; de Jong *et al.* 2000; Rose *et al.* 2002). Our short census period does not allow us to assess the annual variability of survival in *C. thyrsoides*, however, survival rates of 70-95% at seedling size, and > 90% during vegetative rosette stage are very high compared to short-lived monocarpic species (Kachi & Hirose 1985; Sletvold 2005; Rees *et al.* 1999). For long-lived monocarps little data on seedling survival and adult mortality is available and both very low (Augspurger 1985) and high adult mortality rates (Young 1990) have been documented. The probability of survival in *C. thyrsoides* increased with plant size at Furka Pass, whereas in the Schynige Platte population $s(x)$ slowly decreased but was greater than 90% (Fig. 1c). Given there were relatively few seedlings at the SP site during the census period the higher survival rates there are not surprising. We would therefore predict a similar survival distribution as in the FU population when seedlings form a more representative part of the data set. Our findings of high survival rates stress the overall importance of survival for

the evolution of flowering strategies in *C. thyrsoides* (see below) and for long-lived monocarpic species in general.

Reproductive output in *C. thyrsoides* increased proportionally with plant size (Fig. 1d); the slope of the relationship between seed production and size was not significantly different from 1 on a log-log scale. Similar relationships between size/biomass and fecundity have been found in short-lived (Metcalf *et al.* 2003) as well as long-lived monocarps (Young 1984; Augspurger 1985). However, fecundity and seedling recruitment are often decoupled. We demonstrated that despite the seed production varying between 0-68,000 seeds per m^2 the number of new recruits was remarkably constant: this represents an extreme and highly stabilizing form of density dependence which is also observed in short-lived monocarps (Rees *et al.* 1999; Rose *et al.* 2002; Rees *et al.* 2006). Microsite-availability seems therefore to play a key role in the population dynamics of *C. thyrsoides*. This assumption is corroborated by simulating the effect of different establishment rates, p_e, on the population growth parameters, λ and R_0 (Fig. 2). The results revealed dramatic population increase with increasing p_e in agreement with dramatic increases in population size observed in populations along road shoulders and abandoned construction sites that are located in close vicinity to the "stable" study population (Kuss *et al.* 2007). Considerable spatial (relief and substrate heterogeneity, competition) and temporal (seasonal rainfall, successional stage) variability in microsite-availability characterizes habitats of short- and long-lived monocarpic species alike (Augspurger 1985; Young & Augspurger 1991; Holderegger 1997; Körner 2003). The scale and frequency of this spatiotemporal variability potentially determines the persistence of a population and should be manifested through longevity-related difference in demographic functions between short- and long-lived monocarpic species.

LONGEVITY-RELATED DIFFERENCES

Size-dependent flowering combined with a substantial variance about the growth curve leads to a considerable generational overlap the longer lived a species is. In this context, increasing longevity provides a buffer, allowing populations to persist even when the conditions for recruitment are unsuitable in some years as it is frequently the case at high altitude. A consequence of poor recruitment is a lower number of young individuals than would be predicted from the stable age-distribution. This effect accounts for the difference between observed age-distribution, i.e. single-season herb chronology data, and the predicted stable age-distribution at Schynige Platte (Fig. 3c). At the same site the observed age-distribution for

flowering plants (Fig. 3d) was very close to the predicted stable age-distribution. This reflects the fact that only large plants flower and the distribution of large plants is relatively insensitive to variation in recruitment.

We showed that the flowering strategy of *C. thyrsoides* was very close to the evolutionary stable strategy (ESS) and consequently there is only weak selection on the flowering strategy (Fig. 6). The fact that we can predict the flowering strategy observed in the field with data from only a three year study is extremely encouraging. However, this does not mean that other factors not included in the model are unimportant. For example, one might think that because the models used here did not include temporal variation, and yet allowed accurate prediction of the flowering strategy, that temporal variation was not an important determinant of the flowering strategy. The results presented in Rees *et al.* (2004) demonstrate that this is not the case because the effects of different forms of temporal variation may maintain genetic diversity for variable flowering size.

The elasticity analyses demonstrate the importance of survival-growth transitions, $P(y,x)$, for population growth. In both populations the relative contribution of survival-growth (FU:81%; SP: 78%) and fecundity (FU:19%; SP: 22%) elasticities to λ were remarkably similar. In short-lived monocarps, survival-growth transitions are typically more important than fecundity. For example, in *Onopordum illyricum*, Ellner & Rees (2006) found survival-growth vs. fecundity transitions to contribute to 75% vs. 25% to λ, and in *Carlina vulgaris*, Childs *et al.* (2003) calculated a contributions as 66% vs. 34%. The three species show similar patterns of size-specificity of the survival-growth and fecundity elasticities (Fig. 5). It seems therefore likely that increasing importance of survival-growth transitions is related to increased longevity.

In our IPMs we did not incorporate recruitment from a persistent seed bank since neither field nor experimental observations indicated any seed dormancy. Inclusion of a smaller fraction of seed that delayed germination would result in small decreases in λ and R_0, the magnitude of which depends on the probability of seed death. However, the inclusion of a seed bank has no effect on the ESS flowering strategy in a constant environment model because it affects all flowering strategies equally (Rees *et al.* 2006). In a stochastic environment a persistent seed bank could potentially have a significant effect but where models have be built (for *Carlina vulgaris* and *Carduus nutans*; Rees *et al.* 2006) the impact of a seed bank on the ESS flowering strategy was small.

COMPLICATING FACTORS

Some aspects of the biology of monocarpic plants were not included in our IPMs but may never the less be important and merit further study. These are 1) the genetic regulation of size-dependent flowering, 2) the formation of sister rosettes as a result of damage to the apical meristem, and 3) the role of herbivory.

Several studies have shown that substantial genetic variance exists in natural populations for the threshold size of flowering. For example, in *Cynoglossum officinale* and *Senecio jacobaea* experimental selection for early flowering thresholds in the parent generation led to a substantial decrease of flowering size ranges in the offspring generation (Wesselingh & de Jong 1995; Wesselingh & Klinkhamer 1996). Comparable studies with long-lived monocarps are currently missing and in the case of *C. thyrsoides* we have only weak indications from a common garden experiment that half-sibs synchronize flowering even if grown at two different altitudes (P. Kuss, unpublished). However, reliable estimates on the genetic basis of flowering time could not be obtained.

The second complicating factor is the formation of sister-rosettes as a result of damage to the apical meristem. Depending on the study system, sister-rosettes can become independent plants such that genets behave like perennials (e.g. *Saxifraga cotyledon*, *S. mutata*; Webb & Gornall 1989) or more often sister rosettes are autonomous with respect to flowering, but will die together in case one sister-rosette has completed seed set. In either case monocarpic plants can sometimes be encountered with two or more flowering stalks and hereby considerably increase an individual's fecundity. In *C. thyrsoides* about 10% of all plants formed sister-rosettes during the vegetative stage and these lived from one to several years. In extreme cases sister rosettes seemed to disappear and be replaced two years later by a new sister rosette. In the current IPMs we excluded individuals with more than one rosette due to the insufficient permanent plot data and the numerous peculiarities in their behaviour.

Thirdly, herbivory can influence every aspect of plant performance and in *C. thyrsoides* substantial herbivory was only observed by leafroller moth larvae, *Cochylis pallidana,* and two weevil species, *Miarus* cf. *graminis* and *M.* cf. *abeillei*, that fed on immature seeds and could cause complete reproductive failure. Seed-predation however was highly variable in space and time and was never observed in the permanent plots so that we did not incorporate pre-dispersal seed-predation in our IPMs.

CONCLUSION

With the time-lagged IPM we were able to predict accurately, using the ESS approach, the relationship between plant size and the probability of flowering, and also the distributions of sizes and ages at flowering of *C. thyrsoides* in the field. This demonstrates that a good understanding of the demography of long-lived monocarpic species can be obtained using data collected over relatively short periods, and demonstrates the flexibility and power of the integral projection approach.

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1 *R code for the time-lagged integral projection model*
Appendix S2 *Time delays and smoothness*
Appendix S3 *Computing R_0 in a time delay model*
Appendix S4 and Figure S1 *Bootstrap detail*
Appendix S5 *Sensitivity and elasticity in time delay models*

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Appendix S1 *R code for the time-lagged integral projection model*

```
######################################################################
#
#              Time-Lagged Integral Projection Model
#
#               Study species: Campanula thyrsoides
#
#              Last update: Sunday, 13.07.2007
#
######################################################################

rm(list=ls(all=TRUE))

library(nlme)
library(MASS)

#================== Required Input files ====================
#
# 1. "ct.ipm.txt"
#       columns:        n == Unique number for each individual
#                       site == Population abbreviation (here: FU == Furka Pass, SP == Schynige Platte)
#                       year.t == here: 2004 or 2005
#                       nl.t == number of rosette leaves in year t
#                       nl.t1 == number of rosette leaves in year t+1
#                       ll.t == length of longest rosette leave in year t
#                       ll.t1 == length of longest rosette leave in year t+1
#                       surv == survival: 1 == yes, = == no
#                       flow == flowering: 1 == yes, = == no
#                       ros == number of rosettes in individual
#
# 2. "ct.IPM.fecundity.txt"
#       columns:        n == Unique number for each individual
#                       site == Population abbreviation (here: FU == Furka Pass, SP == Schynige Platte)
#                           year.t == here: 2004 or 2005
#                       ll.t == length of longest rosette leave in year t
#                       nl.t == number of rosette leaves in year t
#                       si.t1 == seeds per individual in year t+1
#                       brows.t1 == browsed in year t+1 (here: 1 ==Yes, 0 == No)
#                       ros == number of rosettes in individual
#                       size.t == size in year t
#
# 3. "CT.IPM.seedlings.txt"
#       columns:        n == Unique number for each individual
#                       site ==  Population abbreviation (here: FU == Furka Pass, SP == Schynige Platte)
#                       year.t == here: 2004 or 2005
#                       ll.t ==  length of longest rosette leave in year t
#                       nl.t ==  number of rosette leaves in year t
#                       size.t == size in year t
#
# 4. "IPM.establishment.data.txt"
#       columns:        site == Population abbreviation (here: FU == Furka Pass, SP == Schynige Platte)
#                        plot == Number of plot
#                       year.t == here: 2004 or 2005
#                       seeds.t == seed production in each plot
#                       sdl.t1 == seedlings in year t+1 in each plot
#
# 5. "ipm.saf.data.txt"
#        columns:       n == Unique number for each individual
#                       site == Population abbreviation (here: FU == Furka Pass, SP == Schynige Platte)
```

```
#                   flo == Flowering (here: 1==Yes, 0==NO)
#                   age == Age 2005


#=============================================================
#       Reading in Data
#=============================================================

dataf=data.frame(read.table("ct.ipm.txt",header=T))
      # names(dataf)

attach(dataf)

tmp=order(site[ros==1])

size.t.all=log(nl.t*ll.t)[ros==1][tmp]
size.t1.all=log(nl.t1*ll.t1)[ros==1][tmp]

flow.all=flow[ros==1][tmp]

surv.all=surv[ros==1][tmp]

site.all=site[ros==1][tmp]

site.code.l=c("FU","SP")
pch.code=c(19,1)

all.sizes=c(size.t.all[flow.all==0],size.t1.all[year.t==2005])
all.site=c(site.all[flow.all==0],site.all[year.t==2005])


#####################  Part (I) ###################################
#
#        Fitting models
#
#########################################################################


#=============================================================
#       Calculation: Growth
#=============================================================

size.t=size.t.all[flow.all==0]
size.t1=size.t1.all[flow.all==0]
site.s=site.all[flow.all==0]

test=complete.cases(size.t,size.t1,site.s)

size.t=size.t[test]
size.t1=size.t1[test]
site.s=site.s[test]

# check whether variance structure is needed

fit.grow.gls.1<-gls(size.t1~size.t+site.s,na.action=na.omit,weight=varExp(form=~fitted(.)|site.s),method="ML");
summary(fit.grow.gls.1)

fit.grow.gls<-gls(size.t1~size.t+site.s,na.action=na.omit,weight=varExp(form=~fitted(.)),method="ML");
summary(fit.grow.gls)

fit.grow.gls.0<-gls(size.t1~size.t+site.s,na.action=na.omit,method="ML");
summary(fit.grow.gls.0)

anova(fit.grow.gls.0,fit.grow.gls,fit.grow.gls.1)

# check whether intercept estimate for habitat is needed

fit.grow.gls.0<-gls(size.t1~size.t,na.action=na.omit,weight=varExp(form=~fitted(.)|site.s),method="ML");
```

```
fit.grow.gls.1<-gls(size.t1~size.t+site.s,na.action=na.omit,weight=varExp(form=~fitted(.)|site.s),method="ML");
fit.grow.gls.2<-gls(size.t1~size.t*site.s,na.action=na.omit,weight=varExp(form=~fitted(.)|site.s),method="ML");

anova(fit.grow.gls.0,fit.grow.gls.1,fit.grow.gls.2)

#refit model with size and site main effects, and site specific decreasing variance

fit.grow.gls<-gls(size.t1~site.s+size.t-1,na.action=na.omit,weight=varExp(form=~fitted(.)|site.s),method="ML");

summary(fit.grow.gls)
intervals(fit.grow.gls)

g.intercepts=fit.grow.gls$coef[1:2]
g.slopes=rep(fit.grow.gls$coef[3],2)
var.exp.coef=fit.grow.gls$modelStruct$varStruct
sigma.g=fit.grow.gls$sigma

#=============================================================
#       Plot: Annual growth
#=============================================================

par(mfrow=c(2,2), mar=c(3,3,1,2)+0.1, bty="l",pty="s", cex.main=1, cex.axis=1, cex.lab=1, tck=0.02, mgp=c(2, 0.3 ,0)) ;


plot(size.t,size.t1,
      type="n",
      xlab="Plant size year t",
      ylab="Plant size year t+1",
      xlim=c(2,10),ylim=c(2,10))

for(i in 1:2){
      points(size.t[site.s==site.code.l[i]],size.t1[site.s==site.code.l[i]],pch=pch.code[i])
            abline(g.intercepts[i],g.slopes[i],lty=i)
            abline(0,1,lty=3)
}

text(2.3,10,labels="a)")
legend(7,4.8,
      c("FU: n=735", "SP: n=357"),
      pch=c(19,1),lty=c(1,2),
      bty="n",
      xjust=0)

#=============================================================
#       Calculation: Flowering
#=============================================================

flow.s=flow.all[order(size.t.all)]; length(flow.s)
site.s=site.all[order(size.t.all)]; length(site.s)
size.t=size.t.all[order(size.t.all)]; length(size.t)

store.size.flow=size.t[flow.s==1]
store.site.flow=site.s[flow.s==1]

fit.flow.1=glm(flow.s~size.t*site.s,family=binomial)
fit.flow=glm(flow.s~size.t+site.s,family=binomial)
fit.flow.0=glm(flow.s~size.t,family=binomial)

anova(fit.flow.0,fit.flow,fit.flow.1,test="Chisq")

fit.flow=glm(flow.s~site.s/size.t-1,family=binomial)

f.intercepts=fit.flow$coef[1:2]
f.slopes=c(fit.flow$coef[3:4])

site.flow.SE=summary(fit.flow)$coef[5:6]

#=============================================================
```

```
#       Plot: Flowering
#=============================================================

plot(size.t,flow.s,
      type="n",
      xlab="Plant size year t",
      ylab="Probability of flowering (year t+1)",
      xlim=c(2,10))

n.size<-seq(2,10,length=1000)

for(i in 1:2){
      ncuts<-50
      reps<-ceiling(length(size.t[site.s==site.code.l[i]])/ncuts)
      c.size<-gl(ncuts,reps,length=length(size.t[site.s==site.code.l[i]]))
      pflow<-as.numeric(sapply(split(flow.s[site.s==site.code.l[i]],c.size),mean,na.rm=T))
      msize<-as.numeric(sapply(split(size.t[site.s==site.code.l[i]],c.size),mean,na.rm=T))
      points(msize,pflow,pch=pch.code[i])
      fitted<-exp(f.intercepts[i]+f.slopes[i]*n.size)/ (1+exp(f.intercepts[i]+f.slopes[i]*n.size))
      points(n.size,fitted,type="l",lty=i)
}

text(2.3,1.0,labels="b)")
legend(2, 0.35,
      c("FU: n=827", "SP: n=746"),
      pch=c(19,1),lty=c(1,2),
      bty="n",
      xjust=0)

#=============================================================
#       Calculation: Survival
#=============================================================

surv.s=surv.all[order(size.t.all)]
site.s=site.all[order(size.t.all)]
size.t=size.t.all[order(size.t.all)]

fit.surv.1=glm(surv.s~size.t*site.s,family=binomial)
fit.surv=glm(surv.s~size.t+site.s,family=binomial)
fit.surv.0=glm(surv.s~size.t,family=binomial)

anova(fit.surv.0,fit.surv,fit.surv.1,test="Chisq")

fit.surv=glm(surv.s~site.s/size.t-1,family=binomial)

s.intercepts=fit.surv$coef[1:2]
s.slopes=c(fit.surv$coef[3:4])

#=============================================================
#       Plot: Survival
#=============================================================

plot(size.t,surv.s,
      type="n",
      xlab="Plant size year t",
      ylab="Probability of survival to year t+1",
      xlim=c(2,10))

n.size<-seq(2,10,length=1000)

for(i in 1:2){
      ncuts<-50
      reps<-ceiling(length(size.t[site.s==site.code.l[i]])/ncuts)
      c.size<-gl(ncuts,reps,length=length(size.t[site.s==site.code.l[i]]))
      psurv<-as.numeric(sapply(split(surv.s[site.s==site.code.l[i]],c.size),mean,na.rm=T))
      msize<-as.numeric(sapply(split(size.t[site.s==site.code.l[i]],c.size),mean,na.rm=T))
      points(msize,psurv,pch=pch.code[i])
      fitted<-exp(s.intercepts[i]+s.slopes[i]*n.size)/ (1+exp(s.intercepts[i]+s.slopes[i]*n.size))
```

```
	points(n.size,fitted,type="l",lty=i)
}

text(2.3,1.0,labels="c)")
legend(7,0.35,
	c("FU: n=827", "SP: n=746"),
	pch=c(19,1),lty=c(1,2),
	bty="n",
	xjust=0)

#=================================================================
#	Calculation: Fecundity
#=================================================================

IPM.fecundity=data.frame(read.table("CT.IPM.fecundity.txt",header=T))

	# unbrowsed individuals
size.t.f <- IPM.fecundity$size.t[IPM.fecundity$brows.t1==0]
site.t.f =  IPM.fecundity$site[IPM.fecundity$brows.t1==0]
si.t1.f <- IPM.fecundity$si.t1[IPM.fecundity$brows.t1==0]
nl.t.f <- IPM.fecundity$nl.t[IPM.fecundity$brows.t1==0]

fit.fec=lm(log(si.t1.f)~site.t.f+size.t.f-1)

#=================================================================
#	Plot: Fecundity
#=================================================================

plot(size.t.f,log(si.t1.f),type="n",xlab="Plant size year t",ylab="Viable seeds (log scale) year
t+1",pch=19,xlim=c(2,10),ylim=c(2,10))

for(i in 1:2){
	points(size.t.f[site.t.f==site.code.l[i]],log(si.t1.f)[site.t.f==site.code.l[i]],pch=pch.code[i])
	abline(fit.fec$coef[i],fit.fec$coef[3],lty=i)
}

text(2.3,10,labels="d)")
legend(7,4.75,c("FU: n=25", "SP: n=28"),pch=c(19,1),lty=c(1,2),bty="n",xjust=0)


#=================================================================
#	Seedling sizes
#=================================================================

IPM.seedlings=data.frame(read.table("CT.IPM.seedlings.txt",header=T))

seedlings.size.t=IPM.seedlings$size.t[IPM.seedlings$site!="JU"]

seedlings.site=IPM.seedlings$site[IPM.seedlings$site!="JU"]

tmp=order(seedlings.site)

seedlings.size.t=seedlings.size.t[tmp]

seedlings.site=seedlings.site[tmp]

fit.seedlings=lm(seedlings.size.t~seedlings.site-1)

summary(fit.seedlings)

size.all.plus.seedlings=c(all.sizes,IPM.seedlings$size.t[IPM.seedlings$site!="JU" & IPM.seedlings$year.t!=2003])
site.all.plus.seedlings=c(site.all,IPM.seedlings$site[IPM.seedlings$site!="JU" & IPM.seedlings$year.t!=2003])

IPM.establishment=data.frame(read.table("IPM.establishment.data.txt",header=T))

tmp=order(IPM.establishment$site)

p.est.site=IPM.establishment$site[tmp]
```

```
p.est.seeds.t=IPM.establishment$seeds.t[tmp]
p.est.seedlings=IPM.establishment$sdl.t1[tmp]

est.p.est= sapply(split(p.est.seedlings,p.est.site),sum)/sapply(split(p.est.seeds.t,p.est.site),sum)


#=============================================================
#       Collect parameters
#=============================================================


minsize<-1
maxsize<-12

# Global variables for midpoint rule approximation
# n.big.matrix is the number of mesh points for size, n.age is the number of age classes.
n.big.matrix = 250; n.age = 50; n=n.big.matrix

L= minsize; U= maxsize;

# boundary points b and mesh points y
b = L+c(0:n)*(U-L)/n; y = 0.5*(b[1:n]+b[2:(n+1)]);

# step size for midpoint rule, see equations 4 and 5
h = y[2]-y[1]


store.p.vec=array(NA,dim=c(12,2))

p.vec.names<-rep(NA,12)
p.vec<-rep(0,12);

for(i in 1:2){

      p.vec[1]<- s.intercepts[i]                  ; p.vec.names[1]<-"1st survival param";
      p.vec[2]<- s.slopes[i]                      ; p.vec.names[2]<-"2nd survival param";
      p.vec[3]<- f.intercepts[i]                  ; p.vec.names[3]<-"1st flow param    ";
      p.vec[4]<- f.slopes[i]                      ; p.vec.names[4]<-"2nd flow param    ";
      p.vec[5]<- g.intercepts[i]                  ; p.vec.names[5]<-"ag                ";
      p.vec[6]<- g.slopes[i]                      ; p.vec.names[6]<-"bg                ";
      p.vec[7]<- sigma.g^2                        ; p.vec.names[7]<-"sigma2 growth     ";
      p.vec[8]<- fit.fec$coef[i]                  ; p.vec.names[8]<-"intercept seeds   ";
      p.vec[9]<- fit.fec$coef[3]                  ; p.vec.names[9]<-"slope seeds       ";
      p.vec[10]<- fit.seedlings$coef[i]           ; p.vec.names[10]<-"mean kids size   ";
      p.vec[11]<- summary(fit.seedlings)$sigma^2  ; p.vec.names[11]<-"sigma2 kids size ";
      p.vec[12]<- var.exp.coef[i]                 ; p.vec.names[12]<-"growth variance parameter ";

store.p.vec[,i]=p.vec

}


###################  Part (II) ###################################
#
# Compute the kernel component functions from the fitted models
#
#######################################################################

sx<-function(x,params) {
      u<-exp(params[2]*x+params[1]);
      return(u/(1+u));
}

fx<-function(x,params) {
      u<-exp(params[3]+params[4]*x)
    return(u/(1+u))
}
```

```
gxy<-function(x,y,params) {
      mux<-params[5]+params[6]*x;
      sigmax2<-(params[7])*exp(2*(params[12]*mux))
      sigmax<-sqrt(sigmax2);
      fac1<-sqrt(2*pi)*sigmax;
      fac2<-((y-mux)^2)/(2*sigmax2);
      return(exp(-fac2)/fac1);
}

pxy<-function(x,y,params) { return(sx(x,params)*(1-fx(x,params))*gxy(x,y,params)) }

fxy<-function(x,y,params) {
      nkids<-p.est*exp(params[8]+params[9]*x);
      kidsize.mean<- params[10];
      kidsize.var<- params[11];
      fac1<-sqrt(2*pi)*sqrt(kidsize.var);
      fac2<-((y-kidsize.mean)^2)/(2*kidsize.var);
      f<-sx(x,params)*fx(x,params)*nkids*exp(-fac2)/fac1;
      return(f);
}

#============================================================
#       The 'big matrix' M of size n x n
#============================================================

bigmatrix<-function(n,params) {
# upper and lower integration limits
      L<-minsize; U<-maxsize;

# boundary points b and mesh points y
      b<-L+c(0:n)*(U-L)/n;
      y<-0.5*(b[1:n]+b[2:(n+1)]);

# construct the matrix
    I<-diag(n);
      P<-t(outer(y,y,pxy,params=params))
      B<-t(outer(y,y,fxy,params=params))
      M=array(0,dim=c(2*n,2*n))
      M[1:n,1:n]=P*(U-L)/n
      M[1:n,(n+1):(2*n)]=B*(U-L)/n
      M[(n+1):(2*n),1:n]=diag(n)
      K<-M;
    P<-(U-L)*P/n;
    B<-(U-L)*B/n;
      return(list(matrix=M,kernel=K,meshpts=y,Pmatrix=P,Bmatrix=B,Imatrix=I));
}


R0.calc<-function(n,params){
      M<-bigmatrix(n,params);
      if (any(is.na(M$matrix))){
            ave.R0=NA;
            lam=NA;
            T=NA;
      } else{
            N<-solve(M$Imatrix-M$Pmatrix);
            R<- M$Bmatrix %*% N
            ave.R0<-Re(eigen(R)$values[1]);
            lam<-Re(eigen(M$matrix)$values[1]);
            T=log(ave.R0)/log(lam)
      }

      return(list(lam=lam,ave.R0=ave.R0,T=T))
}

R0.betas<-function(x){
      p.vec[3]<-x;
      nR0<-R0.calc(n.big.matrix,p.vec)
```

```
    return(nR0$ave.R0)
}


#=============================================================
#       Calculation: mean Generation time
#=============================================================


gen.time=rep(NA,2)

for(i in 1:2){
    #if(i==1) p.est= 8.604605e-05 else p.est=0.0001655622  # assuming dd-reg
    if(i==1) p.est= est.p.est[1] else p.est=est.p.est[2]   # actual
    p.vec=store.p.vec[,i]
    tmp=R0.calc(n.big.matrix,p.vec)
    gen.time[i]=tmp$T
    cat("Site ",i," lambda=",tmp$lam," R0=",tmp$ave.R0," Generation time=",tmp$T,"\n")
    cat("ESS intercept ",optimize(R0.betas,c(-100,10),maximum=T,tol=0.01)$maximum,"\n")
}


#=============================================================
#       Calculation: Evolutionary Stable Strategy
#=============================================================


n.test<-100
R0.beta<-array(NA,dim=c(n.test,2));
lam.beta<-array(NA,dim=c(n.test,2));
ESS=rep(NA,2)


#=============================================================
#       Plot: Evolutionary Stable Strategy (see Fig. 6)
#=============================================================

win.graph()
par(mfrow=c(2,2), mar=c(3,3,1,2)+0.1, bty="l",pty="s", cex.main=1, cex.axis=1, cex.lab=1, tck=0.02, mgp=c(2, 0.3 ,0)) ;

for(i in 1:2){
    p.vec=store.p.vec[,i]
    #if(i==1) p.est= 8.604605e-05 else p.est=0.0001655622  # assuming dd-reg
    if(i==1) p.est= est.p.est[1] else p.est=est.p.est[2]   # actual
    if(i==1) beta.flow<-seq(-100,0,length=n.test) else beta.flow<-seq(-50,0,length=n.test);

    for(beta.test in 1:n.test){
        p.vec[3]<-beta.flow[beta.test];
        nR0<-R0.calc(n.big.matrix,p.vec)
        R0.beta[beta.test,i]<-nR0$ave.R0
        lam.beta[beta.test,i]<-nR0$lam
        cat(beta.flow[beta.test],"  ",nR0$ave.R0,"  ",nR0$lam,"\n")
    }

    ESS[i]<-beta.flow[R0.beta[,i]==max(R0.beta[,i])]

    plot(beta.flow,R0.beta[,i],type="n",xlab=expression("Intercept of flowering function " * italic(beta * scriptstyle(0))),
    ylab=expression(italic("R"*scriptstyle(0))))
    min.R0=min(R0.beta[,i]); max.R0=max(R0.beta[,i])
    mean.m2se=fit.flow$coef[i]-2*site.flow.SE[i]
    mean.p2se=fit.flow$coef[i]+2*site.flow.SE[i]

    polygon(c(mean.m2se,mean.p2se,mean.p2se,mean.m2se),c(min.R0,min.R0,max.R0,max.R0), col="grey90",border=0)
    points(beta.flow,R0.beta[,i],type="l")
    abline(h=1)
    points(beta.flow[R0.beta[,i]==max(R0.beta[,i])],max(R0.beta[,i]),pch=19)
    abline(v=fit.flow$coef[i])
    #abline(v=beta.flow[R0.beta==max(R0.beta)])
```

```
    if (i==1) text(locator(1),"a)") else text(locator(1),"c)")

    plot(beta.flow,lam.beta[,i],type="n",xlab=expression("Intercept of flowering function " * italic(beta * scriptstyle(0))),
    ylab=expression(italic(lambda)))
    min.R0=min(lam.beta[,i]);max.R0=max(lam.beta[,i])
    mean.m2se=fit.flow$coef[i]-2*site.flow.SE[i]
    mean.p2se=fit.flow$coef[i]+2*site.flow.SE[i]

    polygon(c(mean.m2se,mean.p2se,mean.p2se,mean.m2se),c(min.R0,min.R0,max.R0,max.R0), col="grey90",border=0)
    points(beta.flow,lam.beta[,i],type="l")
    abline(h=1)
    abline(v=fit.flow$coef[i])
    points(beta.flow[lam.beta[,i]==max(lam.beta[,i])],max(lam.beta[,i]),pch=19)

    if (i==1) text(locator(1),"b)") else text(locator(1),"d)")
}


#=============================================================================================
====#
#       Constructing the component matrices and their transposes
#=============================================================================================
====#


# Put all component matrices into 3-dimensional arrays
P<-array(NA,dim=c(n.big.matrix,n.big.matrix)) #P[j,i,a] will be h*P_{a-1}(x_j,x_i)
B<-array(NA,dim=c(n.big.matrix,n.big.matrix)) #B[j,i,a] will be h*F_{a-1}(x_j,x_i)

stable.dist=array(NA,dim=c(n,n.age,2)); lam.stable.age=rep(NA,2);

for(i in 1:2){
    p.vec=store.p.vec[,i]
    if(i==1) p.est= est.p.est[1] else p.est=est.p.est[2]            #actual
    P<-h*t(outer(y,y,pxy,params=p.vec))
    B<-h*t(outer(y,y,fxy,params=p.vec))


#=============================================================================================
====#
#       Model iteration functions
#=============================================================================================
====#


    Nt=matrix(0,n.big.matrix,n.age); Nt1=Nt; Nt2=Nt       # population now and next year
    iteration=function(Nt1,Nt){
        for(age in 2:n.age){
            Nt2[,age]=P%*%Nt1[,age-1]
            }
        Nt2[,1]=0;

        for(age in 1:n.age){
            Nt2[,1]=Nt2[,1]+B%*%Nt[,age]
            }
        return(Nt2)
        }

#=============================================================================================
====#
#       Start using the model
#=============================================================================================
====#


# Estimate lambda and w by iterating unperturbed matrix
    Nt1=matrix(1,n.big.matrix,n.age);
```

```
    Nt=Nt1
    qmax=1000; lam=1; tol=1.e-8;
    while(qmax>tol) {
        Nt2=iteration(Nt1,Nt);
        qmax=sum(abs(Nt2-lam*Nt1));
        lam=sum(Nt2)/sum(Nt1);

        Nt=Nt1
        Nt1=Nt2

        tot=sum(Nt1+Nt2)

        Nt=Nt/tot
        Nt1=Nt1/tot

        cat(lam,qmax,"\n");
    }

    stable.dist[,,i]=Nt/sum(Nt); lam.stable.age[i]=lam;

}

#=============================================================================#
#  Calculation: Stable distribution and size-dependent total elasticity
#=============================================================================#

stable.dist.flow=stable.dist

p.surv.flow=sx(y,p.vec)*fx(y,p.vec)

for(i in 1:2){
    for(age in 1:n.age){
        stable.dist.flow[,age,i]=stable.dist[,age,i]*p.surv.flow
    }
}

for(i in 1:2){
    stable.dist.flow[,,i]=stable.dist.flow[,,i]/(sum(stable.dist.flow[,,i]))
}

dataf=data.frame(read.table("ipm.saf.data.txt",header=T))
attach(dataf)

flow.age=age.05[flo.05==1]
flow.site=as.numeric(site[flo.05==1])
age=age.05
site=as.numeric(site)

stable.dist.age=array(NA,dim=c(n.age,2))
stable.dist.age.flow=array(NA,dim=c(n.age,2))


#=============================================================================#
#  Plot: Stable age distribution (see Fig. 2)
#=============================================================================#

win.graph()
par(mfrow=c(2,2), mar=c(3,3,1,2)+0.1, bty="l",pty="s", cex.main=1, cex.axis=1, cex.lab=1, tck=0.02, mgp=c(2, 0.3 ,0)) ;

for(i in 1:2){
    hist(age[site==i],breaks=seq(0,50,3),freq=F,col="grey",main="",xlab="Age (years)", ylim=c(0,0.2))
    stable.dist.age[,i]=apply(stable.dist[,,i],2,sum)
    points(0:(n.age-1),stable.dist.age[,i],type="l")
    if (i==1) text(locator(1),"a)") else text(locator(1),"c)")

    hist(flow.age[flow.site==i],breaks=seq(0,50,3),freq=F,col="grey",main="",xlab="Age (years)",ylim=c(0,0.20))
    stable.dist.age.flow[,i]=apply(stable.dist.flow[,,i],2,sum)
    points(0:(n.age-1),stable.dist.age.flow[,i],type="l")
```

```
      if (i==1) text(locator(1),"b)") else text(locator(1),"d)")
}

mean.age.f=rep(NA,2)
mean.size.f=rep(NA,2)

for(i in 1:2){
      mean.age.f[i] <-sum((1:(n.age))*apply(stable.dist.flow[,,i],2,sum))-1;
      cat("Mean flowering age",mean.age.f[i],"\n")
      mean.size.f[i]<-sum(exp(y)*apply(stable.dist.flow[,,i],1,sum));
      cat("Mean flowering size",mean.size.f[i],"\n")
}

stable.dist.size=array(NA,dim=c(n.big.matrix,2))
stable.dist.size.flow=array(NA,dim=c(n.big.matrix,2))


#=============================================================================#
#  Plot: Stable size distribution (see Fig. 3)
#=============================================================================#

win.graph()
par(mfrow=c(2,2), mar=c(3,3,1,2)+0.1, bty="l",pty="s", cex.main=1, cex.axis=1, cex.lab=1, tck=0.02, mgp=c(2, 0.3 ,0)) ;

for(i in 1:2){
      hist(size.all.plus.seedlings[site.all.plus.seedlings==i],freq=T,col="grey",main="",xlab="Plant
size",breaks=seq(1,10.5,0.5))
      stable.dist.size[,i]=sum(!is.na(size.all.plus.seedlings[site.all.plus.seedlings==i]))*apply(stable.dist[,,i],1,sum)/(y[2]-
y[1])/2
      points(y,stable.dist.size[,i],type="l")
      if (i==1) text(locator(1),"a)") else text(locator(1),"c)")

      hist(store.size.flow[store.site.flow==site.code.l[i]],freq=T,col="grey",main="",xlab="Plant size",breaks=seq(2,9.5,0.5))
      stable.dist.size.flow[,i]=sum(!is.na(store.size.flow[store.site.flow==site.code.l[i]]))*apply(stable.dist.flow[,,i],1,sum)/(y[
2]-y[1])/2
      points(y,stable.dist.size.flow[,i],type="l")
      if (i==1) text(locator(1),"b)") else text(locator(1),"d)")
}


#=============================================================================#
#  iterate model with time lag
#=============================================================================#

stable.dist.tl=array(0,dim=c(n,2))
lam.stable.tl=rep(NA,2)
b = L+c(0:n)*(U-L)/n; y = 0.5*(b[1:n]+b[2:(n+1)]);
h = y[2]-y[1]

for(i in 1:2){
      p.vec=store.p.vec[,i]
      if(i==1) p.est= est.p.est[1] else p.est=est.p.est[2]            #actual
      P<-h*t(outer(y,y,pxy,params=p.vec))
      B<-h*t(outer(y,y,fxy,params=p.vec))

      qmax=1000

      Nt=matrix(1/n,n); Nt1=Nt/2;  # population now, next year and the one after

      while(qmax>1e-10) {
            Nt2=P%*%Nt1+B%*%Nt
            qmax=sum(abs(Nt2-lam*Nt1));
            lam=sum(Nt2)/sum(Nt1);
            Nt=Nt1;
            Nt1=Nt2;
            tot=sum(Nt+Nt1)
            Nt=Nt/tot
            Nt1=Nt1/tot
```

```
    }
    stable.dist.tl[,i]=Nt/sum(Nt); lam.stable.tl[i]=lam;
}

lam.stable.age
lam.stable.tl


#=============================================================================#
#  Calculation: sensitivity and elasticity by perturbation P matrix
#=============================================================================#


sen.big.P<-array(NA,dim=c(n,n))        #array to store the results
elas.big.P<-array(NA,dim=c(n,n,2))

for(i in 1:2){
    p.vec=store.p.vec[,i]
    if(i==1) p.est= est.p.est[1] else p.est=est.p.est[2]              #actual

    P<-h*t(outer(y,y,pxy,params=p.vec))
    B<-h*t(outer(y,y,fxy,params=p.vec))


    for(row in 1:n) {              # loop over y values
        # choose x* to maximize e(y,x) for this y value, by scanning across the row
        big.one=which(P[row,]*stable.dist.tl[,i]==max(P[row,]*stable.dist.tl[,i]));

        # perturb the kernel up and down near (y,x*)
        delta=0.1*h*P[row,big.one];
        Pup=P; Pup[row,big.one] = P[row,big.one]+delta/h;
        Pdown=P; Pdown[row,big.one] = P[row,big.one]-delta/h;

        qmax=1; lamup=1; lamdown=1;
        Nt.up<-stable.dist.tl[,i]; Nt1.up<-stable.dist.tl[,i]
        Nt.down<-stable.dist.tl[,i]; Nt1.down<-stable.dist.tl[,i]

        while(qmax>1e-10) {
            Nt2.up=Pup%*%Nt1.up+B%*%Nt.up
            qmax=sum(abs(Nt2.up-lamup*Nt1.up));
            lamup=sum(Nt2.up)/sum(Nt1.up);

            Nt.up=Nt1.up;
            Nt1.up=Nt2.up;
            tot=sum(Nt.up+Nt1.up)
            Nt.up=Nt.up/tot
            Nt1.up=Nt1.up/tot

            Nt2.down=Pdown%*%Nt1.down+B%*%Nt.down

            qmax=qmax+sum(abs(Nt2.down-lamdown*Nt1.down));
            lamdown=sum(Nt2.down)/sum(Nt1.down);

            Nt.down=Nt1.down;
            Nt1.down=Nt2.down;
            tot=sum(Nt.down+Nt1.down)
            Nt.down=Nt.down/tot
            Nt1.down=Nt1.down/tot

            #cat(lamup,lamdown,qmax,"\n");
        }


        sen.big.row<-(lamup-lamdown)/(2*delta) #sensitivity for perturbation at (y,x*)
        sen.big.P[row,]<-(stable.dist.tl[,i]/stable.dist.tl[,i][big.one])*sen.big.row #sensitivity at other x's
        cat(row,big.one,lamup,lamdown," sens=",sen.big.row, "\n")
    }
```

```
    elas.big.P[,,i]=(P/h)*sen.big.P/lam.stable.tl[i];

}

sum(elas.big.P[,,1]*h*h)
sum(elas.big.P[,,2]*h*h)


#=============================================================================#
#  Calculation: sensitivity and elasticity by perturbation B matrix
#=============================================================================#


sen.big.B<-array(NA,dim=c(n,n))        #array to store the results
elas.big.B<-array(NA,dim=c(n,n,2))

for(i in 1:2){
    p.vec=store.p.vec[,i]
    if(i==1) p.est= est.p.est[1] else p.est=est.p.est[2]            #actual

    P<-h*t(outer(y,y,pxy,params=p.vec))
    B<-h*t(outer(y,y,fxy,params=p.vec))

    for(row in 1:n) {              # loop over y values
        # choose x* to maximize e(y,x) for this y value, by scanning across the row
        big.one=which(B[row,]*stable.dist.tl[,i]==max(B[row,]*stable.dist.tl[,i]));

        # perturb the kernel up and down near (y,x*)
        delta=0.1*h*B[row,big.one];
        Bup=B; Bup[row,big.one] = B[row,big.one]+delta/h;
        Bdown=B; Bdown[row,big.one] = B[row,big.one]-delta/h;

        qmax=1; lamup=1; lamdown=1;
        Nt.up<-stable.dist.tl[,i]; Nt1.up<-stable.dist.tl[,i]
        Nt.down<-stable.dist.tl[,i]; Nt1.down<-stable.dist.tl[,i]

        while(qmax>1e-10) {
            Nt2.up=P%*%Nt1.up+Bup%*%Nt.up
            qmax=sum(abs(Nt2.up-lamup*Nt1.up));
            lamup=sum(Nt2.up)/sum(Nt1.up);

            Nt.up=Nt1.up;
            Nt1.up=Nt2.up;
            tot=sum(Nt.up+Nt1.up)
            Nt.up=Nt.up/tot
            Nt1.up=Nt1.up/tot

            Nt2.down=P%*%Nt1.down+Bdown%*%Nt.down

            qmax=qmax+sum(abs(Nt2.down-lamdown*Nt1.down));
            lamdown=sum(Nt2.down)/sum(Nt1.down);

            Nt.down=Nt1.down;
            Nt1.down=Nt2.down;
            tot=sum(Nt.down+Nt1.down)
            Nt.down=Nt.down/tot
            Nt1.down=Nt1.down/tot

            #cat(lamup,lamdown,qmax,"\n");
        }

        sen.big.row<-(lamup-lamdown)/(2*delta) #sensitivity for perturbation at (y,x*)
        sen.big.B[row,]<-(stable.dist.tl[,i]/stable.dist.tl[,i][big.one])*sen.big.row #sensitivity at other x's
        cat(row,big.one," sens=",sen.big.row, "\n")
    }

    elas.big.B[,,i]=2*(B/h)*sen.big.B/lam.stable.tl[i];
```

```
}

sum(elas.big.B[,,1]*h*h)+sum(elas.big.P[,,1]*h*h)
sum(elas.big.B[,,2]*h*h)+sum(elas.big.P[,,2]*h*h)


#=============================================================================#
#  Plot: elasticity (see Fig 5)
#=============================================================================#


win.graph()
par(mfrow=c(2,2), mar=c(3,3,1,2)+0.1, bty="l",pty="s", cex.main=1, cex.axis=1, cex.lab=1, tck=0.02, mgp=c(2, 0.3 ,0)) ;

zmax=max(elas.big.P,elas.big.B)

image(y,y,t(elas.big.P[,,1]),xlab="Plant size year t",ylab="Plant size year
t+1",col=grey(1:300/300),gamma=0.2,zlim=c(0,1.1*zmax));
      contour(y,y,t(elas.big.P[,,1]),add=T,cex=3,levels = c(0.01,0.05,0.1,0.15,0.2,0.25,0.3));
      text(locator(1),"a)",col="white")

image(y,y,t(elas.big.B[,,1]),xlab="Plant size year t-1",ylab="Plant size year
t+1",col=grey(1:300/300),gamma=0.2,zlim=c(0,1.1*zmax));
      contour(y,y,t(elas.big.B[,,1]),add=T,cex=3,levels = c(0.01,0.05,0.1,0.15,0.2,0.25,0.3));
      text(locator(1),"b)",col="white")

image(y,y,t(elas.big.P[,,2]),xlab="Plant size year t",ylab="Plant size year
t+1",col=grey(1:300/300),gamma=0.2,zlim=c(0,1.1*zmax));
      contour(y,y,t(elas.big.P[,,2]),add=T,cex=3,levels = c(0.01,0.05,0.1,0.15,0.2,0.25,0.3));
      text(locator(1),"c)",col="white")

image(y,y,t(elas.big.B[,,2]),xlab="Plant size year t-1",ylab="Plant size year
t+1",col=grey(1:300/300),gamma=0.2,zlim=c(0,1.1*zmax));
      contour(y,y,t(elas.big.B[,,2]),add=T,cex=3,levels = c(0.01,0.05,0.1,0.15,0.2,0.25,0.3));
      text(locator(1),"d)",col="white")


#=============================================================================#
#  Calculation: new big matrix approximation
#=============================================================================#


M.tl=array(0,dim=c(2*n,2*n))
lam.stable.bm=rep(NA,2)
R0.stable.bm=rep(NA,2)

for(i in 1:2){
      p.vec=store.p.vec[,i]
      if(i==1) p.est= est.p.est[1] else p.est=est.p.est[2]            #actual
      if(i==1) p.vec[3]=-58.67228 else p.vec[3]= -26.25266            #actual

      P<-h*t(outer(y,y,pxy,params=p.vec))
      B<-h*t(outer(y,y,fxy,params=p.vec))

      M.tl[1:n,1:n]=P
      M.tl[1:n,(n+1):(2*n)]=B
      M.tl[(n+1):(2*n),1:n]=diag(n)

      lam.stable.bm[i]=Re(eigen(M.tl)$values[1]);

      #R0
      M.P=array(0,dim=c(2*n,2*n))
      M.B=array(0,dim=c(2*n,2*n))

      M.P[1:n,1:n]=P
      M.P[(n+1):(2*n),1:n]=diag(n)

      M.B[1:n,(n+1):(2*n)]=B
```

```
    N<-solve(diag(2*n)-M.P);
    R<- M.B %*% N
    R0.stable.bm[i]<-Re(eigen(R)$values[1]);

    T.stable.mb <-log(R0.stable.bm)/log(lam.stable.bm)      # Generation time
}


lam.stable.bm      # Lambda
R0.stable.bm       # Net reproductive rate
T.stable.mb        # Generation time


#=============================================================
#       Simulate Lambda, R0 and T with varying p.est
#=============================================================


seq.start <- 0.00001
seq.end <- 0.001
seq.by <- 0.00001
p.est.seq <- seq(seq.start,seq.end,by=seq.by)
n.p.est <- length(p.est.seq)

p.est.FU <- 0.00016
p.est.SP <- 0.00078

Res.p.est <- matrix(0,n.p.est,8)
dimnames(Res.p.est) <- list(1:n.p.est,c("p.est","p.est","l.FU","l.SP","Ro.FU","Ro.SP","T.FU","T.SP"))

#new big matrix approximation

M.tl=array(0,dim=c(2*n,2*n))
lam.stable.bm=rep(NA,2)
R0.stable.bm=rep(NA,2)

for (k in 1:n.p.est){
    for(i in 1:2){
        p.est= p.est.seq[k]
        p.vec=store.p.vec[,i]
        P<-h*t(outer(y,y,pxy,params=p.vec))
        B<-h*t(outer(y,y,fxy,params=p.vec))
        M.tl[1:n,1:n]=P
        M.tl[1:n,(n+1):(2*n)]=B
        M.tl[(n+1):(2*n),1:n]=diag(n)
        lam.stable.bm=Re(eigen(M.tl)$values[1]);

    # R0
        M.P=array(0,dim=c(2*n,2*n))
        M.B=array(0,dim=c(2*n,2*n))
        M.P[1:n,1:n]=P
        M.P[(n+1):(2*n),1:n]=diag(n)
        M.B[1:n,(n+1):(2*n)]=B
        N<-solve(diag(2*n)-M.P);
        R<- M.B %*% N
        R0.stable.bm<-Re(eigen(R)$values[1]);

    # Generation time
        T.stable.bm <-log(R0.stable.bm)/log(lam.stable.bm)

    # Filling in of result matrix
        Res.p.est[k,i] <- p.est
        Res.p.est[k,i+2] <- lam.stable.bm
        Res.p.est[k,i+4] <- R0.stable.bm
        Res.p.est[k,i+6] <- T.stable.bm
    }
```

```
}

Res.p.est

#=============================================================================#
#  Plot: Simulated Lambda, R0 and T with varying p.est (see Figure 4)
#=============================================================================#


win.graph
par(mfrow=c(1,3), mar=c(3,3,1,2)+0.1, bty="l",pty="s", cex.main=1, cex.axis=1, cex.lab=1, tck=0.02, mgp=c(2, 0.3 ,0)) ;

plot(Res.p.est[,1],Res.p.est[,3],type="n",xlab=expression(paste(italic(p)[e])),ylab=expression(lambda))
      lines(Res.p.est[,1],Res.p.est[,3],lty=1)
      lines(Res.p.est[,1],Res.p.est[,4],lty=2)
      points(p.est.FU,1.05, pch=19, cex=2.0); points(p.est.SP,1.17, pch=1, cex=2.0);
      legend(0.0006,1,c("FU", "SP"),pch=c(19,1),lty=c(1,2),bty="n",xjust=0,cex=1.5)

plot(Res.p.est[,1],Res.p.est[,5],type="n",xlab=expression(paste(italic(p)[e])),ylab=expression(paste(italic(R[0]))))
      lines(Res.p.est[,1],Res.p.est[,5],lty=1)
      lines(Res.p.est[,1],Res.p.est[,6],lty=2)
      points(p.est.FU,1.67, pch=19, cex=2.0); points(p.est.SP,4.97, pch=1, cex=2.0);

plot(Res.p.est[,1],Res.p.est[,7],type="n",xlab=expression(paste(italic(p)[e])),ylab=expression(italic(T)),ylim=c(10,13))
      lines(Res.p.est[,1],Res.p.est[,7],lty=1)
      lines(Res.p.est[,1],Res.p.est[,8],lty=2)
      points(p.est.FU,10.82, pch=19, cex=2.0); points(p.est.SP,10.37, pch=1, cex=2.0);


#=============================================================================#
#  Bootstrap lambda, R0 and generation time (see Appendix S4)
#=============================================================================#


n.boot=5000
dem.stats=array(NA,dim=c(n.boot,3,2))
boot.ESS=array(NA,dim=c(n.boot,2))

M.tl=array(0,dim=c(2*n,2*n))

for(b.samp in 1:n.boot){

      #growth

      size.t=size.t.all[flow.all==0]
      size.t1=size.t1.all[flow.all==0]
      site.s=site.all[flow.all==0]
      test=complete.cases(size.t,size.t1,site.s)
      size.t=size.t[test]
      size.t1=size.t1[test]
      site.s=site.s[test]
      sample.boot=c(sample(1:735,replace=T),735+sample(1:357,replace=T))
      size.t.boot=size.t[sample.boot]
      size.t1.boot=size.t1[sample.boot]
      fit.grow.gls.boot<-gls(size.t1.boot~site.s+size.t.boot-
1,na.action=na.omit,weight=varExp(form=~fitted(.)|site.s),method="ML");
      g.intercepts.boot=fit.grow.gls.boot$coef[1:2]
      g.slopes.boot=rep(fit.grow.gls.boot$coef[3],2)
       var.exp.coef.boot=fit.grow.gls.boot$modelStruct$varStruct
      sigma.g.boot=fit.grow.gls.boot$sigma

      #survival and flowering

      sample.boot=c(sample(1:827,replace=T),827+sample(1:746,replace=T))
      size.t.boot=size.t.all[sample.boot]
      flow.all.boot=flow.all[sample.boot]
      surv.all.boot=surv.all[sample.boot]
      fit.flow.boot=glm(flow.all.boot~site.all*size.t.boot-1,family=binomial)
```

```
  f.intercepts.boot=fit.flow.boot$coef[1:2]
  f.slopes.boot=c(fit.flow.boot$coef[3],fit.flow.boot$coef[3]+fit.flow.boot$coef[4])
  fit.surv.boot=glm(surv.all.boot~site.all*size.t.boot-1,family=binomial)
  s.intercepts.boot=fit.surv.boot$coef[1:2]
  s.slopes.boot=c(fit.surv.boot$coef[3],fit.surv.boot$coef[3]+fit.surv.boot$coef[4])

  #fecundity

  sample.boot=c(sample(1:25,replace=T),25+sample(1:28,,replace=T))
size.t.f.boot=size.t.f[sample.boot]
si.t1.f.boot=si.t1.f[sample.boot]
fit.fec.boot=lm(log(si.t1.f.boot)~site.t.f+size.t.f.boot-1)

  #seedlings

  sample.boot=c(sample(1:573,replace=T),573+sample(1:123,replace=T))
  seedlings.size.t.boot=seedlings.size.t[sample.boot]
  fit.seedlings.boot=lm(seedlings.size.t.boot~seedlings.site-1)

  #p.est

  sample.boot=c(sample(1:70,replace=T),70+sample(1:42,replace=T))
  p.est.seeds.t.boot=p.est.seeds.t[sample.boot]
  p.est.seedlings.boot=p.est.seedlings[sample.boot]
  est.p.est.boot= sapply(split(p.est.seedlings.boot,p.est.site),sum)/sapply(split(p.est.seeds.t.boot,p.est.site),sum)


  for(i in 1:2){
        p.vec[1]<- s.intercepts.boot[i]
        p.vec[2]<- s.slopes.boot[i]
        p.vec[3]<- f.intercepts.boot[i]
        p.vec[4]<- f.slopes.boot[i]
        p.vec[5]<- g.intercepts.boot[i]
        p.vec[6]<- g.slopes.boot[i]
        p.vec[7]<- sigma.g.boot^2
        p.vec[8]<- fit.fec.boot$coef[i]
        p.vec[9]<- fit.fec.boot$coef[3]
        p.vec[10]<- fit.seedlings.boot$coef[i]
        p.vec[11]<- summary(fit.seedlings.boot)$sigma^2
        p.vec[12]<- var.exp.coef.boot[i]

        if(i==1) p.est= est.p.est.boot[1] else p.est=est.p.est.boot[2]

        P<-h*t(outer(y,y,pxy,params=p.vec))
        B<-h*t(outer(y,y,fxy,params=p.vec))

        M.tl=array(0,dim=c(2*n,2*n))
        M.tl[1:n,1:n]=P
        M.tl[1:n,(n+1):(2*n)]=B
        M.tl[(n+1):(2*n),1:n]=diag(n)

        lam=Re(eigen(M.tl)$values[1]);

  #R0

        M.P=array(0,dim=c(2*n,2*n))
        M.B=array(0,dim=c(2*n,2*n))

        M.P[1:n,1:n]=P
        M.P[(n+1):(2*n),1:n]=diag(n)

        M.B[1:n,(n+1):(2*n)]=B

        N<-solve(diag(2*n)-M.P);
        R<- M.B %*% N
        R0<-Re(eigen(R)$values[1]);

  #generation time
```

```
            T=log(R0)/log(lam)
            boot.data=c(lam,R0,T)

            dem.stats[b.samp,,i]=boot.data

            if(any(!is.na(boot.data))){
                  boot.ESS[b.samp,i]<-optimize(R0.betas,c(-150,10),maximum=T,tol=0.01)$maximum
            } else boot.ESS[b.samp,i]=NA
      }
      cat("sample ",b.samp,"\n")
}

getStats=function(x){
      ci.normal.app=c(mean(x)-1.96*sd(x),mean(x)+1.96*sd(x))
      res=c(mean(x,na.rm=T),quantile(x,p=c(0.025,0.5,0.975),na.rm=T),ci.normal.app)
      return(res)
      }

for(i in 1:2){
      cat("site ",site.code.l[i],"\n")
      print(apply(dem.stats[,,i],2,getStats))
      print(getStats(boot.ESS[,i]))
}


#=============================================================================#
#  Plot: Bootstrap lambda, R0 and generation time (see Appendix S4)
#=============================================================================#


win.graph()
par(mfrow=c(2,4), mar=c(3,3,1,2)+0.1, bty="l",pty="s", cex.main=1, cex.axis=1, cex.lab=1, tck=0.02, mgp=c(2, 0.3 ,0)) ;

main.titles=c(expression(italic(lambda)),expression(italic(R[0])),"Generation time, T",expression("ESS " * italic(beta *
scriptstyle(0))))

for(i in 1:2){
      for(j in 1:3){
            hist(dem.stats[,j,i][dem.stats[,j,i]<30],xlab=main.titles[j],col="grey",main="")
            abline(v=mean(dem.stats[,j,i],na.rm=T))
            abline(v=median(dem.stats[,j,i],na.rm=T),col="blue")
            abline(v=quantile(dem.stats[,j,i],p=0.025,na.rm=T),col="red")
            abline(v=quantile(dem.stats[,j,i],p=0.975,na.rm=T),col="red")
      }

      hist(boot.ESS[,i],col="grey",xlab=main.titles[4],main="")
      abline(v=mean(boot.ESS[,i],na.rm=T))
      abline(v=median(boot.ESS[,i],na.rm=T),col="blue")
      abline(v=quantile(boot.ESS[,i],p=0.025,na.rm=T),col="red")
      abline(v=quantile(boot.ESS[,i],p=0.975,na.rm=T),col="red")
      abline(v=f.intercepts[i],col="green",lwd=2)
}

lam.diff=dem.stats[,1,1]-dem.stats[,1,2]
mean(lam.diff,na.rm=T)
quantile(lam.diff,p=c(0.025,0.5,0.975),na.rm=T)
R0.diff=dem.stats[,2,1]-dem.stats[,2,2]
mean(R0.diff,na.rm=T)
quantile(R0.diff,p=c(0.025,0.5,0.975),na.rm=T)
T.diff=dem.stats[,3,1]-dem.stats[,3,2]
mean(T.diff,na.rm=T)
quantile(T.diff,p=c(0.025,0.5,0.975),na.rm=T)

#save(dem.stats,boot.ESS,file="c:\\temp\\ipm sheffield\\bootstrap samples.Rdata")
```

Appendix S2 *Time delays and smoothness*

The time-delay model is represented symbolically by the operator $\mathbf{K} = \begin{pmatrix} \mathbf{P} & \mathbf{F} \\ \mathbf{I} & \mathbf{0} \end{pmatrix}$ where **I** denotes the identity operator, with the "matrix multiplication" interpretation

$$\mathbf{K}\begin{pmatrix} n_1 \\ n_2 \end{pmatrix} = \begin{pmatrix} \mathbf{P} & \mathbf{F} \\ \mathbf{I} & \mathbf{0} \end{pmatrix}\begin{pmatrix} n_1 \\ n_2 \end{pmatrix} = \begin{pmatrix} \mathbf{P}n_1 + \mathbf{F}n_2 \\ n_1 \end{pmatrix} \quad 1$$

K does not satisfy the assumptions of Ellner & Rees (2006) because the identity operator is not represented by a smooth kernel acting on the population state distribution (the same is true for any trait that changes deterministically). This situation is rescued, however, by the fact that the second-iterate is represented by smooth kernels:

$$\mathbf{K}^2 = \begin{pmatrix} \mathbf{P} & \mathbf{F} \\ \mathbf{I} & \mathbf{0} \end{pmatrix}\begin{pmatrix} \mathbf{P} & \mathbf{F} \\ \mathbf{I} & \mathbf{0} \end{pmatrix} = \begin{pmatrix} \mathbf{P}^2 + \mathbf{F} & \mathbf{PF} \\ \mathbf{P} & \mathbf{F} \end{pmatrix} \quad 2$$

.

The general theory in Ellner & Rees (2006, Appendix C) therefore applies to $\mathbf{K}^2$, implying the existence of stable population structure, long-term growth rate, etc. as required for the analyses in this paper, so long as some iterate of $\mathbf{K}^2$is either positive or *u*-bounded. In our *Campanula* model both **P** and **F** are positive, so the first iterate ($\mathbf{K}^2$ itself) is positive.

Appendix S3 *Computing R_0 in a time delay model*

Here we show that for computing R_0 the time delay can be ignored, i.e. R_0 is the dominant eigenvalue of $\mathbf{F}(\mathbf{I}-\mathbf{P})^{-1}$ where **F** and **P** are the fecundity and survival/growth operators defined by the kernels *F* and *P*. See Appendix B of Ellner & Rees (2006) for a discussion of the conditions required for the existence of R_0.

For the time-delay model the fecundity and growth operators are (using the notation of equation 1, Appendix S2)

$$\mathbf{F}_0 = \begin{bmatrix} \mathbf{0} & \mathbf{F} \\ \mathbf{0} & \mathbf{0} \end{bmatrix}, \quad \mathbf{P}_0 = \begin{bmatrix} \mathbf{P} & \mathbf{0} \\ \mathbf{I} & \mathbf{0} \end{bmatrix}$$

So R_0 is the dominant eigenvalue of the next-generation operator

$$\mathbf{R} = \begin{bmatrix} \mathbf{0} & \mathbf{F} \\ \mathbf{0} & \mathbf{0} \end{bmatrix}\left(\mathbf{I} - \begin{bmatrix} \mathbf{P} & \mathbf{0} \\ \mathbf{I} & \mathbf{0} \end{bmatrix}\right)^{-1} = \begin{bmatrix} \mathbf{0} & \mathbf{F} \\ \mathbf{0} & \mathbf{0} \end{bmatrix}\begin{bmatrix} \mathbf{I}-\mathbf{P} & \mathbf{0} \\ -\mathbf{I} & \mathbf{I} \end{bmatrix}^{-1} \quad . \quad 3$$

The inverse in equation 3 can be computed. For a 2×2 matrix with scalar entries and $a \neq 0$,

$$\begin{bmatrix} a & 0 \\ -1 & 1 \end{bmatrix}^{-1} = \begin{bmatrix} a^{-1} & 0 \\ a^{-1} & 1 \end{bmatrix}.$$

Similarly for 3 we have

$$\begin{bmatrix} \mathbf{I}-\mathbf{P} & \mathbf{0} \\ -\mathbf{I} & \mathbf{I} \end{bmatrix}^{-1} = \begin{bmatrix} (\mathbf{I}-\mathbf{P})^{-1} & \mathbf{0} \\ (\mathbf{I}-\mathbf{P})^{-1} & \mathbf{I} \end{bmatrix} \quad 4$$

which can be verified directly. Using equation 4 in equation 3 gives

$$\mathbf{R} = \begin{bmatrix} \mathbf{F}(\mathbf{I}-\mathbf{P})^{-1} & \mathbf{F} \\ \mathbf{0} & \mathbf{0} \end{bmatrix} \quad . \quad 5$$

Any eigenvector of **R** is in the range of **R** and therefore is of the form $n = \begin{bmatrix} n_1 \\ 0 \end{bmatrix}$. Consequently n_1 is an eigenvector of $\mathbf{F}(\mathbf{I}-\mathbf{P})^{-1}$ with the same corresponding eigenvalue as *n*. Conversely, if n_1 is an eigenvector of $\mathbf{F}(\mathbf{I}-\mathbf{P})^{-1}$ then *n* is an eigenvector of **R** with the same eigenvalue. Thus **R** and $\mathbf{F}(\mathbf{I}-\mathbf{P})^{-1}$ have the same set of eigenvalues and the same dominant eigenvalue R_0.

Appendix S4 *Bootstrap details*

To obtain estimates of the uncertainty associated with the various measures of population growth, generation time and ESS flowering strategy we bootstrapped the data (Fig S1). Where site effects were significant we randomly sampled with replacement individual data pairs from within each site and fitted the site specific models to the combined data from both sites. This was done for the growth, survival and flowering probabilities, fecundity and the distribution of seedling sizes. For the probability of seedling establishment we resampled the data from each quadrate within each site then calculated p_e by dividing the total number of seedlings by the total seed production the previous year. Thus each complete bootstrapped sample allows an IPM to be constructed for both sites, and so site specific demographic parameters (i.e. λ, R_0 etc) can be estimated. The bootstrapped distributions for λ and the generation time, *T,* are symmetrical and approximately follow a normal distribution. In these cases the error involved in using the normal approximation to the 95% confidence interval is small (<1%).

To test if the various measures of population growth (λ and R_0) and generation time varied between sites we calculated the difference between the calculated statistics for each site, using each bootstrapped dataset.

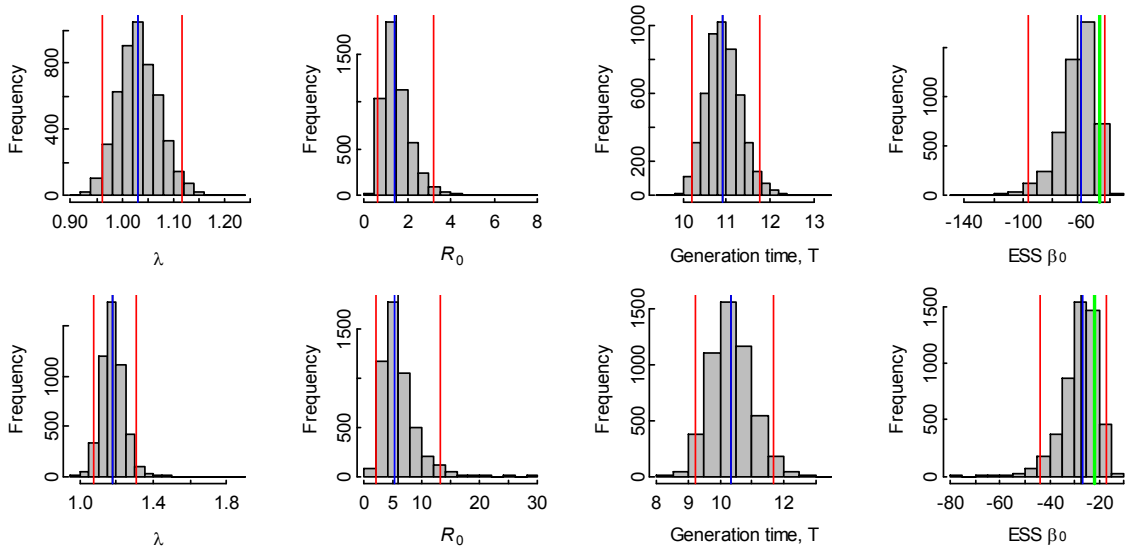

Figure S1. Bootstrapped distributions of population growth rate, λ, net reproductive rate, R_0, generation time, *T*, and intercept β_0 of the evolutionary stable flowering strategy (ESS) for *Campanula thyrsoides*. Red lines are the 2.5 and 97.5 percentiles (95% confidence intervals), black lines the means, blue the medians and the estimated values of β_0 are shown in green. Top row refers to FU, bottom to SP.

Appendix S5 *Sensitivity and elasticity in time delay models*

The fecundity operator **F** in the *Campanula thyrsoides* model is the composition of two operators, **F=SM** where **M** represents transitions from current rosettes to the (unmeasured) flowering stage, and **S** represents seedling production by flowering plants. Using these operators the model can be written without time delays as

$$\begin{pmatrix} n_r(t+1) \\ n_f(t+1) \end{pmatrix} = \begin{pmatrix} \mathbf{P} & \mathbf{S} \\ \mathbf{M} & \mathbf{0} \end{pmatrix} \begin{pmatrix} n_r(t) \\ n_f(t) \end{pmatrix} \qquad 6$$

where n_r and n_f are the size-distribution functions for rosettes and flowering plants, respectively. This form of the model is suitable for standard sensitivity/elasticity calculations. Elasticity values correspond to a fractional increase in one rate within the projection operator (a single entry in the case of a matrix model, or a local perturbation whose support is shrunk to 0 in the case of an integral model, see pp. 417-418 in Ellner & Rees 2006). For model 6 we have the usual result that the total elasticity equals 1 (total elasticity being the sum of all elasticities for a matrix model, and the integral of the elasticity function for an integral model), so that individual elasticities can be interpreted as fractional contributions to population growth.

The interpretation of elasticities is more complicated for the time-delay form of the model, equation 1, for two reasons. First, any one elasticity for **F,** computed directly by making a fractional local perturbation and recomputing λ, corresponds to perturbing multiple locations in **S** and **M** because $F(y,x) = \int S(y,z)M(z,x)dz$. The correspondence is not unique: a given change in $F(y,x)$ could result from many different perturbations to *S* and *M*. So any elasticity for **F** represents the aggregate contribution of all pathways, through flowering plants of different sizes, from a rosette of size *y* to a

new recruit of size x. And each step in each of those pathways contributes to multiple rosette-to-recruit transitions (e.g., $M(z,x)$ contributes to the elasticity of $F(y,x)$ for *all* values of y).

Second, the elasticities for **P** and **F** relate to different time intervals, and as a result it is not the case that the total elasticity computed by local perturbations in **P** and **F** equals 1. If we increase **P, S** and **M** by a factor $1+\varepsilon$ in 6, then λ increases by the same factor – this is why total elasticity equals 1 for a model without time delays. When **S** and **M** are both increased by a factor $1+\varepsilon$, **F** increases by a factor $(1+\varepsilon)^2$, which to leading order equals $1+2\varepsilon$. Consequently, the total elasticity for the time-delay model will only equal 1 if the perturbations to **F** are doubled, or equivalently, if elasticities are computed in the usual way and then doubled. The same should be done when comparing the total contributions of **P** and **F** to population growth rate: before making the comparison, the elasticities for **F** (computed in the usual way) should be doubled.

Chapter 8

The Biological Flora of Central Europe: *Campanula thyrsoides* L.

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Abstract

Campanula thyrsoides is one of the few monocarpic perennials of temperate alpine mountain ecosystems and native to the European Alps and adjacent mountains ranges. Individuals are rare but locally abundant and the species is protected in most of the Alpine countries. The exceptionally yellow-flowering member of the Campanulaceae grows preferably in alpine meadows with a moderate disturbance regime on limestone or carbonate bearing schists. Traditionally regarded as a rosette-forming "biennial", individuals usually flower after 8 years and grow considerably older at higher altitudes. This article reviews the taxonomy, morphology, distribution, ecology, life cycle, population biology, and genetics of this species as well as its status in the European countries.

Key words: Alpine, ecology, monocarpic perennial, semelparity, species biology

Taxonomy and Morphology

Taxonomy

Campanula thyrsoides L. (Campanulaceae) – Yellow bellflower
Synonyms: *C. thyrsoidea* L. (cf. Linneus, 1767)
C. thyrsoïdea L. (cf. Hegi, 1975)

Please note that *C. thyrsoidea* Lapeyrouse has been used as synonym for *C. speciosa* Pourret from the Pyrenees (Picot de Lapeyrouse, 1813).

Two subspecies have been recognised that differ in morphological and palynological characteristics as well as in ecology and distribution (Podlech, 1964; Caramiello et al., 2002-2003). Nevertheless, transitional forms exist within and outside of their geographic contact zones (Podlech, 1964; Kuss, pers. obs.) and the two subspecies are sometimes regarded as altitudinal vicariants (Tomažič, 1941).

C. thyrsoides L. subsp. *thyrsoides*
Basionym: *C. thyrsoides* Linnaeus 1753, Sp. Pl.: 167

Common names: Gewöhnliche Strauß-Glockenblume – Campanule en thyrse – Campanula gialla – Šopasta zvončica – Brunzina püschlada

C. thyrsoides L. subsp. *carniolica* (Sündermann) Podlech 1964, Ber. Bayer. Bot. Ges. 37: 110
Basionym: *C. thyrsoides* L. var. *carniolica* Sündermann 1925, Allg. Bot. Zeitschr. 26/27: 23

Common names: Krainer Strauß-Glockenblume – Campanule de Carniole – Campanula carniolica – Kranjska zvončica – Žućkastobijela zvončika

In the following text we will refer to *C. thyrsoides* subsp. *thyrsoides* as *C.* thyrsoides* and likewise to *C. thyrsoides* subsp. *carniolica* as *C.* carniolica*. Where information applies to both subspecies we use *C. thyrsoides*. Please note that *C.* thyrsoides* is by far the more common subspecies and therefore much more is known.

The genus *Campanula* includes approximately 400-600 species and occurs almost exclusively in the temperate zone of the Northern Hemisphere. Highest species diversity in the genus is found in the mountain ranges of the Eastern Mediterranean and the Caucasus (Fedorov and Kovanda, 1978; see also Meusel and Jäger, 1992). Within the European Alps 42 *Campanula* species are recognized of which 20 are endemic or subendemic (Aeschimann et al., 2005). *Campanula thyrsoides* is positioned into the large section *Campanula* s. str. which is characterized by dehiscent capsules with basal pores, and thus separated from the second section *Rapunculus* with lateral or sub-apical pores (Fedorov and Kovanda, 1978). This morphological classification and *C. thyrsoides*' position therein has been confirmed by molecular investigations (Eddie et al., 2003). For earlier classifications see de Candolle (1830), Schönland (1889-1894), and Fiori (1927). *C. thyrsoides* is further placed into the subsect. *Involucratae* characterized by sessile flowers that are crowded in heads, whorls or clusters and flowers are enveloped by large bracts which facilitate secondary diaspore presentation. Species of this subsection are usually more or less densely pubescent, e.g. *C. glomerata*, *C. cephalotes* Nakai, *C. cervicaria* L., *C. macrochlamys* Boiss. et Huet, *C. macrostachya* Waldst. et Kit. ex Willd., *C. petraea* L., *C. spicata* L., *C. stricta* L. (Fedorov, 1957).

The genus *Campanula* hosts a range of life-forms, from annuals to long-lived polycarpic perennials. The monocarpic (= semelparous or hapaxanth) strategy of *C. thyrsoides* is also found in other species from various subsections (Shulkina, 1980) so that a monophyletic origin of semelparity is unlikely. Pluriennial monocarpic *Campanula* species are e.g. *C. alpina* Jacq., *C. cervicaria* L., *C. macrochlamys* Boiss. et Huet, *C. macrostachya* Waldst. et Kit. ex Willd., *C. medium* L., *C. mirabilis* Albov, *C. moesiaca* Velen., *C. patula* subsp. *patula* L., *C. patula* subsp. *costae* (Willk.) Nyman, *C. patula* subsp. *jahorinae* (K. Maly) Greuter & Burdet, *C. petraea* L., *C. pyramidalis* L., *C. sibirica* subsp. *sibirica* L., *C. spicata* L. and *C. transsilvanica* Schur ex Andrae (Jäger, 2000; Bernini et al., 2002.

Morphology

The two subspecies differ mainly with respect to inflorescence height and spike density (*C.* thyrsoides*: 10-40 cm and dense, *C.* carniolica*: 40-100 cm and basal flowers rather lax), length of floral bracts (*C.* thyrsoides*: as long as corolla and not canaliculate, *C.* carniolica*: twice as long as corolla and canaliculate), canaliculate leaves in *C.* carniolica* (Sündermann, 1925; Podlech, 1964), as well as a number of (not further characterized)

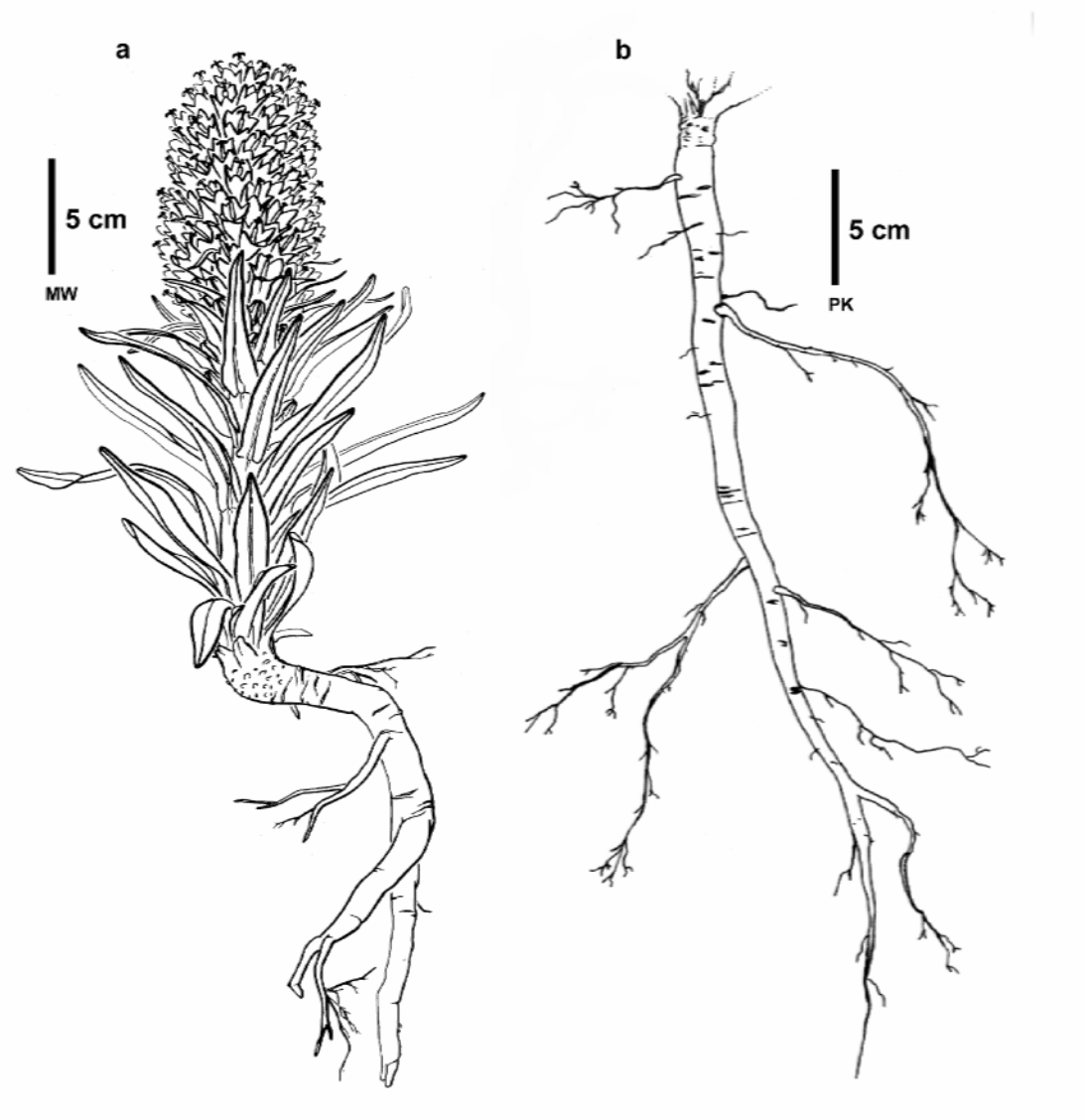

Figure 1. *Campanula thyrsoides*. (a) Habitus at flowering and (b) root profile of vegetative plant, 8-years old, in rock crevice, Furka Pass, Switzerland.

palynological features (Caramiello et al., 2002-2003). All further information applies to both subspecies. For color images of the two subspecies see Aeschimann et al. (2005).

Campanula thyrsoides has a thick, fleshy and whitish tap root, commonly 20-40 cm long, but occasionally protruding to a depth of 1 m (Figure 1). The roots as well as all other parts of the plant contain milky and sticky latex. The root architecture is fairly plastic with respect to branching of the main tap root and frequency of fine roots (< 1 mm) as a response to the diverse soil conditions. In water-logged or rocky soils the roots often grow almost horizontally spreading in several directions, while in mesic to dry as well as in deep soils the

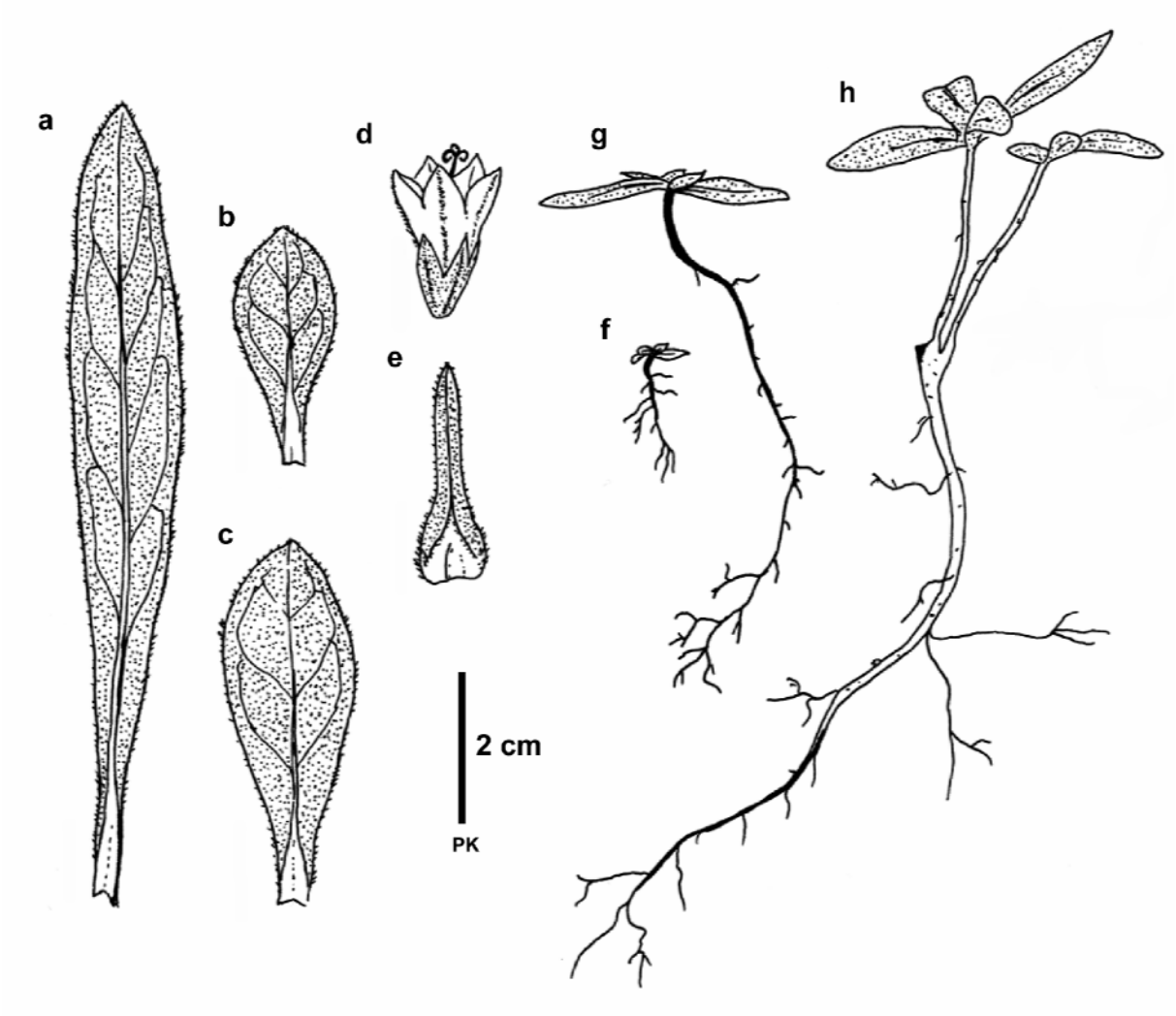

Figure 2. *Campanula thyrsoides*. (a) Spring leaf; (b,c) summer leaves; (d) flower; (e) bract; (f) seeling; (g) 1-year old vegetative plant; (h) 4-year old vegetative plant, note: two sister rosettes.

roots are mostly unbranched and vertically expanding. The root collar of flowering plants measures around 11 mm (range: 4-20 mm).

Vegetative individuals form rosettes with a maximum diameter of 45 cm and 20-30 leaves (Figure 2 f-h and Figure 3; see Life-Cycle). Annual rosette growth is characterized by two phases of foliar development with two types of leaves. Spring leaves are oblong to elliptical, entire margined, sometimes undulate, with an obtuse to acute apex, cuneate leaf base that slowly tapers into the petiole and are densely covered with stiff whitish-brownish hairs (Figure 2 a). The midrib is well developed. Depending on age, leaves measure 6 × 4 mm to 225 × 35 mm. Summer leaves are 2-4 times shorter, obovate to oblanceolate and are formed at the center of the spring rosette (Figure 2 b,c). The large and variable number of

spring and summer rosette leaves wither over winter leaving a tunica of leave bases. The species can therefore be regarded as semi-evergreen (sensu Grime et al., 1988).

The flowering stalk is erect, 10-100 cm tall (subspecies specific), angular, hirsute, densely foliose with sessile, alternate, 20 × 4 mm to 120 × 16 mm long, oblong to obovate, sometimes linear leaves. The inflorescence is composed of 50 to 200 flowers forming a dense or moderately lax spike (Figure 1 a). Flowers are erect, either single or arranged in cymules of 2-5 sessile flowers in the axils of short and pointed linear bracts (Figure 2 e). Calyx lobes are green, linear to lanceolate, acute, approximately half as long as the corolla and densely tomentose with stiff white hairs. The corolla is bell-shaped, 15-25 mm long, with villose nerves and a faint pleasant scent (Figure 2 d). Unlike the majority of *Campanula* species which are blue-flowered, *C. thyrsoides* has yellow to pale-yellow flowers. In the European Alps, this color peculiarity is only shared by *C. albicans* Jacq. (white) and *C. petraea* L. (yellow; Aeschimann et al., 2005) with both species also belonging to subsection Involucratae (Fedorov and Kovanda, 1978). No biochemical information on the yellow color pigmenation is available to date (cf. Hegnauer, 1962-2001). Adamovic (1909) mentioned *C. thyrsoides* to have blue flowers throughout the Balcans. However, this has never been confirmed in the literature or from herbarium specimen. The flowers are hermaphroditic with five stamens, white filaments, yellow anthers, a single central style and three stigmatic lobes. The style is partly covered with retractable pollen-collecting hairs. These are more or less uniform in length, regularly arranged and have ascending tips that point toward the stigma (Nyman, 1993a).

The yellow pollen grains are monadic, 3- or 4-zonoporate and porous, radially symmetric, isopolar, oblate-sphaeroidal to subprolate in shape, appearing subcircular to circular in equatorial view (33.2 µ) and peritreme in polar view (32.7 µ). The surface is rugulate with spines which are uniformely distributed (base: 1.8 µ, heigth: 2.2 µ, distance: 2.0 µ). The aperture is situated in equatorial position, lolongate, circular or lalongate in shape and with indistinct annulus (P: 4.9 µ, E: 4.5 µ, mesoporium: 20.1 µ). The exine measures 2.2 µ, comprised of sexine: 1.2 µ and nexine: 1.0 µ (Caramiello et al., 2002-2003).

Capsules are erect, three-loculate, each locus containing approximately 40-60 seeds (see Reproduction). Well developed viable seeds are brownish, 1.05 ± 0.15 mm long, compressed, sometimes with a rudimentarily developed winged margin. Less developed seeds are of similar size but lighter in color, thinner and rarely viable. Early aborted seeds are blackish to brownish round granules. Capsules open at the base with an outward retraction of the lower capsule tissue. Seeds are trapped by the withered concave bracts and the felted

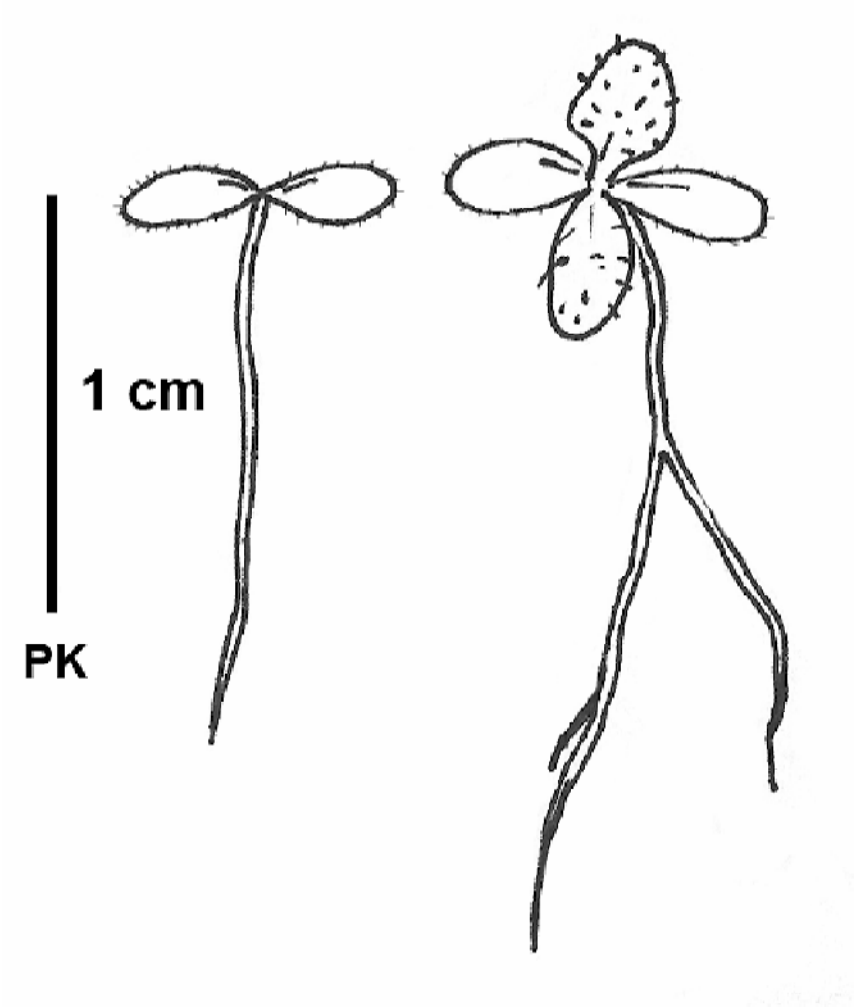

Figure 3. *Campanula thyrsoides*. Seedlings: 2 and 4 weeks after germination on wet filter paper.

unicellular hairs of bracts, calyx and corolla. The withered infructescence remains upright as a winter stander for as long as a year. The seed arresting mechanism in combination with the lignified infructescence lead to a secondary diaspore presentation and an aboveground seed pool, essentially similar to the monocarpic *Campanula cervicaria* (Often, 1999) or the perennial *C. glomerata* (Emig and Leins, 1996). Seeds are only dispersed when strong winds, rain or animals shake the seeds out of the seed trap (see Reproduction).

In case the apical meristem of the flowering stalk is damaged due to herbivory or mowing, secondary shoots with pedicelate terminal flower cymules can be formed in the axils of the lower leaves. This leads to a peculiar, rather bushy habitus of the plant sometimes referred to as *C. thyrsoides* L. var. *glomerata* Saut. (Schiebler, 1935) or *C. thyrsoides* f. *putata* (Hegi, 1975).

Distribution and habitat requirements

Geographical distribution

C. thyrsoides is native to the European Alps and adjacent mountain ranges to the East (Balcans and Dinarids) and North-West (Jura) and occurs in France, Italy, Switzerland, Lichtenstein, Austria, Germany, Slovenia, Croatia, Bosnia-Hercegovina and Bulgaria. An updated distribution map is presented in Figure 4, a generalized distribution map can be found in Aeschimann et al. (2005), and country specific information is available in print or electronically: Bosnia-Hervegovina (Šoljan, 2001), Croatia (Nikolić, 2006), France (Brisse et al., 1996-2006), Germany (Haeupler and Schönfelder, 2003), Switzerland (Welten and Sutter, 1982; Wohlgemuth, 1993). Considerable uncertainty exists with regard to present occurrences in Bulgaria since *C. thyrsoides* has not been collected there after 1930 (Anchev, pers. comm.). Also, it is unclear whether *C. thyrsoides* still exists in Serbia or whether historical records actually refer to a closely related taxon (Josifovic, 1974; Jäger, 2000).

The two subspecies have separated distribution ranges with *C.* * *thyrsoides* occurring in the Western, Central and North-Eastern Alps as well as in the Jura mountains, thus having a cottian-helvetic-(north-noric) distribution pattern (Figure 5: west of Line C). *C.* * *carniolica* is only described from the South-Eastern Alps and the adjacent Dinarids and Balcans, hence a carnian distribution (Figure 5 east of Line C; Jäger, 2000). *C.* * *thyrsoides* is mostly found in subalpine to alpine habitats between 1600-2200 m a.s.l. with lowest occurrences reported around 1010 m a.s.l. (Oytal, Bavarian Alps; Hegi, 1975) and 1040 m a.s.l. (Chapfensee, Switzerland; Seitter, 1989) and highest locations in Switzerland at 2800 m a.s.l. (Col de Sorebois; Becherer, 1956) and 2900 m a.s.l. (Val Mora; Brunies, 1906). In contrast, *C.* * *carniolica* grows preferentially in submontane to montane habitats between 400-1800 m a.s.l. with lowest reported occurrences around 300 m a.s.l. (Kozje, Slovenia; Hegi, 1975; Jäger, 2000). The morphological (and ecological) differentiation of the two subspecies has been hypothesized to be a result of isolation and survival in different glacial refugia during the Quaternary (Jäger, 2000). The review of Schönswetter et al. (2005) outlines current knowledge on locations of glacial refugia in the Alps and in this context, *C.* * *carniolica* is predominately found below the permanent snow line of the last glaciation and outside of previously glaciated terrain (Figure 5: east of Line C). This may suggest that *C.* * *carniolica* has persisted in refugia at lower altitudes than *C.* * *thyrsoides*. In contrast, *C.* * *thyrsoides* was

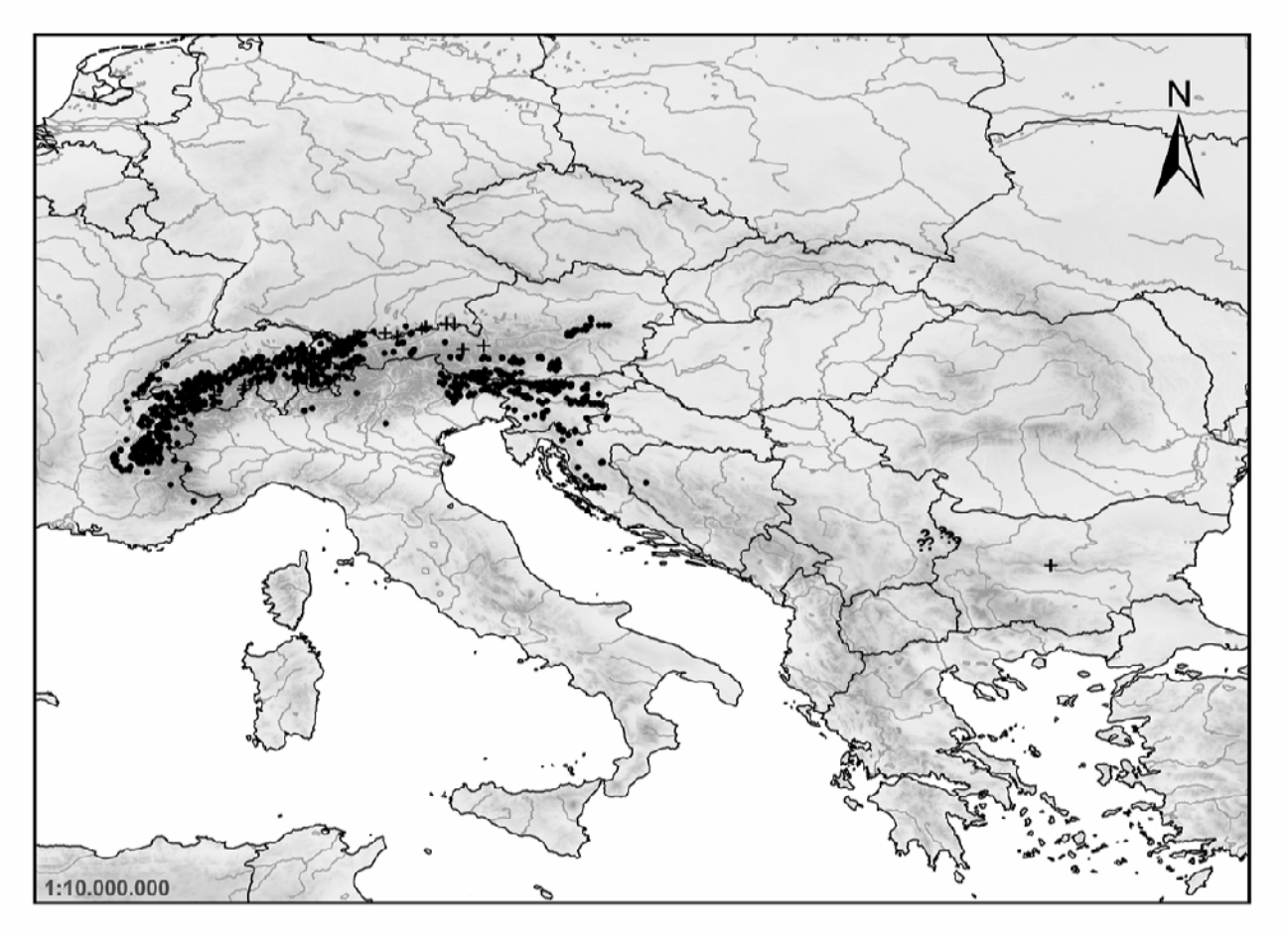

Figure 4. Geographic distribution of *Campanula thyrsoides*. Black dots: confirmed locations; **?**: questionable locations, +: not recently confirmed.

able to recolonize previously glaciated terrain and is seldom found in peripheral refugia below the last glacial permanent snow line. Populations of *C.* thyrsoides* are genetically but not morphologically differentiated along the prominent biogeographical line in Western Switzerland (Figure 5: Line A, see Genetic Data, cf. Schönswetter et al., 2005). No genetic analysis of populations across Line B (Figure 5) exists so far.

However, *C.* thyrsoides* does not seem to be restricted to the Central and Western Alps but is also mentioned from locations throughout the area of *C.* carniolica* (e.g. Kovačić, 2004). For example, *C.* thyrsoides* is reported from the Bosnian Klekovača mountain (Abadžić and Šilić, 1990), from Slovenian sites above 1600 m a.s.l. (Wraber, pers. comm.) and also characterizations from Bulgaria seem to refer to this subspecies (Stojanov and Kitanov, 1966). To further complicate things, large individuals (60 cm) with a lax inflorescence and long, caniculate bracts have been found in Switzerland at 1700 m a.s.l. and thus could belong to *C.* carniolica* (Kuss, pers. obs.). It remains therefore unclear to which

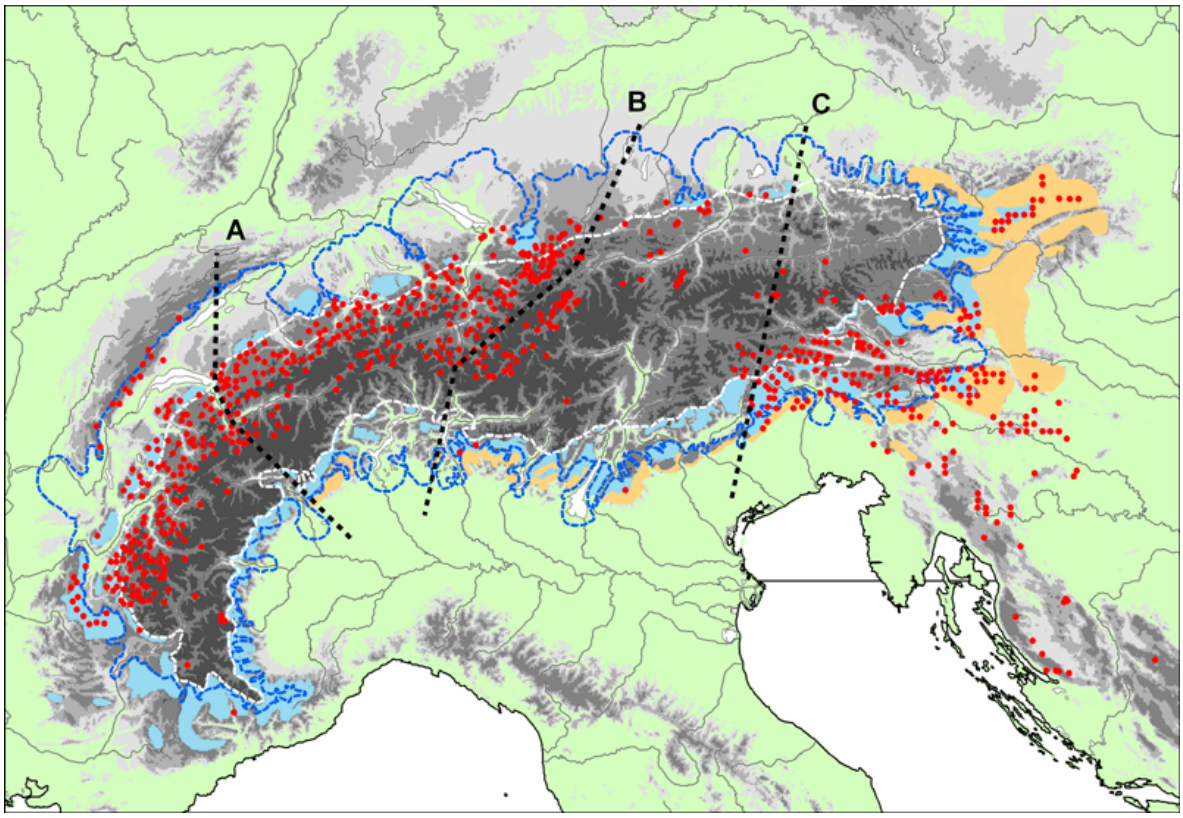

Figure 5. Geographic distribution of *Campanula thyrsoides* within the European Alps and adjacent Dinarids (South-East) and Jura mountains (North-West). Dashed lines represent proven (Line A: see Genetic Data) or probable (Lines B, C) genetic separation of *C. thyrsoides'* populations as a result of Quaternary isolation and coinciding largely with major biogeographical lines (cf. Schönswetter et al., 2005). Line C separates the two subspecies: *C.* * *thyrsoides* – west of C, *C.* * *carniolica* – east of C. Red dots: *C. thyrsoides* populations; blue areas: alpine refugia; orange areas: subalpine refugia; blue line: maximum ice extent; white line: snow line. Map modified after Schönswetter et al. (2005) by E. Welk (Halle), basemaps by A. Tribsch (Salzburg)

extent glacial isolation has driven morphological and ecological differentiation and further, whether morphological differences are to be seen as a response to altitude.

Habitat

C. thyrsoides is a basiphilous species growing in shallow to deep soils derived from limestone, carbonate-bearing schists, dolomite or gypsum, and exceptionally granite with average pH-values between 5 to 8 (Béguin, 1972; Schubiger-Bossard, 1988; Vittoz, 1998) and little to moderate nitrogen availability (Béguin, 1972). Typical soils are e.g. Lithic Leptosols

[Syrosem] and Rendzic Leptosols [Eurendzina], Calcaric Regosols [Pararendzina] and Phaeozems [Mullgley], Gleyic Cambisols [Gley-Braunerde], Calcaric Cambisols [Pararendzina] and Mollic Cambisols [Lockerbraunerde], seldom Folic Histosols [Tangelrendzina] or Haplic Histosols [Niedermoortorf] (e.g. Béguin, 1972; Vittoz, 1998); nomenclature follows World Reference Base for Soils and [German classification], Scheffer and Schachtschabel, 1998). *C. thyrsoides* can tolerate a wide range of water regimes from xeric/mesic to mesic/wet conditions and snow cover for 6-7 months. Plants are usually found in open and sunny habitats on moderately (0-10°) to steeply inclined (>60°) E-, S- or W-facing slopes. The species seems to be adapted to and requires a moderate disturbance regime (meso- to euhemerobic) which creates microsites for successful germination of seeds. Disturbances may either be topography-induced processes, e.g. slope movement, or trampling by animals. A detailed experimental and modelling study to elucidate the importance of disturbance for the establishment of C.* thyrsoides is in preparation. Ellenberg's indicator values represent only in part the ecological amplitude of the species: L8, T2, K4, F5, R7, N4, S0 (Ellenberg, 1996). The categories describe the ecological behaviour of a species with respect to light (L), temperature (T), continentality (K), moisture (F), reaction/soil acidity (R), nitrogen availability (N) as well as salinity (S) and the ordinal values '1' to '9' indicate low to high values of a particular category. The ordinal value '0' refers to a 'non-applicable' category.

Communities

Most populations of *C.* thyrsoides* are found in natural and anthropogenic species-rich calcareous grasslands from treeline ecotone into the alpine belt but plants also occur in tall-herb communities of avalanche shoots and forest edges, alpine fens, forest clearings, screes, precipices, river banks and road shoulders. The wide ecological amplitude of the species, or maybe the existence of the particular niche in many habitats, poses some problems with respect to a phytosociological characterization such that occurrences are reported in a variety of associations, alliances, orders and classes (Table 1). *C.* thyrsoides* has been repeatedly regarded as a characteristic species of the alliance Caricion ferrugineae which comprises species-rich sub-alpine and alpine grasslands on calcareous substrates (Oberdorfer, 1977; Grabherr and Mucina, 1993; Ellenberg, 1996; Wilmanns, 1998) and this alliance is referred to as the "syntaxonomic optimum" of the subspecies (Aeschimann et al., 2005). Older literature refers to *C.* thyrsoides* as being characteristic for the association Caricetum

Table 1. Syntaxonomical units with occurrences of *Campanula thyrsoides* subsp. *thyrsoides*. Nomenclature follows Grabherr and Mucina (1993), Mucina et al. (1993) with additions from Julve (1988–2006) and Oberdorfer (1977). Bold: reported occurences; underlined: *C.* thyrsoides* is characteristic species; entries in [] denote geographic origin of relevés (A: Alps; CH: Switzerland, J: Jura), Syn.: Synonym.

Seslerietea albicantis Oberd. 1978 corr. Oberd. 1990
Seslerietalia coeruleae Br.-Bl. in Br.-Bl. et Jenny 26
Seslerion coeruleae Br.-Bl. in Br.-Bl. et Jenny 26
Seslerio-Caricetum sempervirentis Br.-Bl. in Br.-Bl. et Jenny 26 (Reinalter, 2004) [**A**]
Alchemillo hoppeanae-Seslerietum caeruleae Luquet et Aubert 1930
Syn: **Alchemillo conjunctae-Seslerietum albicantis** (Vittoz, 1998) [**J**]
Veronico fruticulosae-Agrostietum sideritetosum Béguin 1972 (Béguin, 1972) [**J**]
Caricion ferruginae G. Br.-Bl. et J. Br.-Bl. 1931(Oberdorfer, 1977; Grabherr, 1993) [**A**]
Caricetum ferrugineae Lüdi 21(Oberdorfer, 1977; Reinalter, 2004) [**A**]
Trifolio thalii-Festucetum nigricantis Br.-Bl. in Br.-Bl. et Jenny 1926 corr. Grabherr [**A**]
Syn: Trifolio thalii-Festucetum violaceae Br.-Bl. in Br.-Bl. et Jenny 1926
Syn: **Festucetum violaceae** (Reinalter, 2004) [**CH**]
Peucedano-Laserpitietum J.-L. Richard 1977 (Richard, 1977; Theurillat, 1991) [**CH**]
Senecioni-Caricetum sempervirentis J.-L. Richard 1977 (Richard, 1977; Gillet et al., 1994) [**CH**]
Calamagrostion variae Sillinger 1929
Origano-Calamagrostietum variae Lippert ex Thiele 1978
Syn: **Laserpitio latifoliae-Calamagrostietum variae** (Kuhn 37, Moor 57) Th. Müll. 61(Béguin, 1972; Oberdorfer, 1978; Vittoz, 1998) [**J**]
Syn: **Calamagrostidetum variae** Gams 1927 (Gams, 1927) [**CH**]
Campanulo thyrsoideae-Laserpitietum latifoliae Béguin 1972 (Béguin, 1972; Theurillat, 1991)
Mulgedio-Aconitetea Hadač et Klika in Klika et Hadač 1944
Adenostyletalia G. Br.-Bl. et J. Br.-Bl. 1931
Adenostylion alliariae Br.-Bl. 1926
Cicerbitetum alpinae Bolleter 1921 subassoc. **rhaponticetosum** (Kuss, unpublished) [**CH**]
Koelerio-Corynephoretea Klika in Klika et Novák 1941
Alysso-Sedetalia Moravec 1967
Allyso allysoidis-Sedion albi Oberd. et T. Müller in T. Müller 1961
Sedo acris-Poetum alpinae Royer 1985 (Royer, 1985; Vittoz, 1998) [**J**]
Festuco-Brometea Br.-Bl. et R. Tx. ex Klika et Hadač 1944
Brometalia erecti Br.-Bl. 1936
Bromion erecti Koch 1926
Onobrychido viciifoliae-Brometum T. Müller 1966
Syn: Carlino acaulis-Brometum Oberd. 1957
Syn: **Gentiano vernae-Brometum erecti** (Vittoz, 1998) [**J**]
Ranunculo montani-Agrostietum capillaris (Vittoz, 1998) [**J**]
Anemono nemorosae-Caricetea sylvaticae Gillet 1986 (see Gallandat et al., 1995)
Mercurialietalia perennis Gillet 1986
Actaeo spicatae-Mercurialion perennis Gillet 1986
Valeriano montani-Polygonatetum verticillati Gillet [**J**]
Seslerio caeruleae - Mercurialion perennis Gillet 1986
Melampyro sylvatici-Calamagrostietum variae Vittoz 1998 (Vittoz, 1998) [**J**]
Thlaspietea rotundifolii Br.-Bl.1948
Epilobietalia fleischeri Moor 1958
Salicion incanae Aichinger 1933
Epilobietum fleischeri Frey 1922 (Schubiger-Bossard, 1988) [**CH**]

Table 1. continued

Molinio-Arrhenatheretea R. Tx. 1937 em. R. Tx. 1970
Poo alpinae-Trisetetalia Ellmauer et Mucina
Polygono-Trisetion Br.-Bl. et R. Tx. ex Marschall 1947 nom. inv.
Trisetum flavescentis Rübel 1911(Reinalter, 2004) [**CH**]

Scheuchzerio-Caricetea fuscae R. Tx. 1937
Caricetalia davallianae Br.-Bl. 1949
Caricion davallianae Klika
Caricetum davallianae Dutoit 1924 (Reinalter, 2004; Kuss unpublished.) [**CH**]

Caricetea curvulae Br.-Bl. 1948
Caricetalia curvulae Br.-Bl. in Br.-Bl. et Jenny 1926
Festucion variae Guinochet 1938
Hypochoerido uniflorae-Festucetum paniculatae Hartl in Theurillat 1989
Syn: **Laserpitio-Helictotrichetum pratensis** (Reinalter, 2004) [**CH**]
Syn: **Laserpitio-Avenetum pratensis** (Braun-Blanquet, 1969) [**CH**]
Dracocephalo-Potentilletum Br.-Bl. 1969 (Reinalter, 2004) [**CH**]

Carici rupestris-Kobresietea bellardii Ohba 1974
Oxytropido-Kobresietalia Oberdorfer ex Albrecht 1969
Oxytropido-Elynion Br.-Bl. 1949
Elynetum myosuroidis Rübel 1911 (Reinalter, 2004) [**CH**]

Table 2. Syntaxonomical units with occurrences of *Campanula thyrsoides* subsp. *carniolica*. Nomenclature and structure as in Table 1. Slo: Slovenia.

Thlaspietea rotundifolii Br.-Bl.1948
Thlaspietalia rotundifolii Br.-Bl. in Br.-Bl. et Jenny 1926 em. Seibert 1977
Petasition paradoxi (Aeschimann et al., 2005) [**A**]

Festuco-Brometea Br.-Bl. et R. Tx. ex Klika et Hadač 1944
Brometalia erecti Br.-Bl. 1936
Syn: **Seslerietalia juncifoliae** Ht 1930 (Abadžić and Silić, 1990) [**Slo**]
Bromion erecti Koch 1926
Scabioso hladnikianae-Caricetum humilis Tomažič 1941 (Tomažič, 1941) [**Slo**]
Bromo-Plantaginetum mediae Horvat 1931 (Šugar, 1972) [**Slo**]

Querco-Fagetea Br.-Bl. et Vilieger 1937
Quercetalia pubescentis Br.-Bl. (1931 n. nud.) 1932
Ostryo-Carpinion orientalis Ht. 1954 emend. 1958
Cytisantho-Ostryetum Wraber 1960 (Wraber, 1960; Horvat et al., 1974) [**Slo**]

ferrugineae (e.g. Braun-Blanquet, 1948) but this indicator status has been transferred to the alliance level. However, given the predominant occurrences of *C.* thyrsoides* in different meadow types within the order Seslerietalia coerulea (Table 1) we suggest transferring the subspecies to the order or class level. Additionally, it appears more appropriate to speak of a syntaxonomic optimum in this order than assigning characterer status as the species is present in a range of different dry meadows communities.

Among the many syntaxa listed in Table 1, it is noteworthy that *C.* thyrsoides* is name-giving species for a plant community restricted to the Jura mountains, Campanulo

thyrsoideae-Laserpitietum latifoliae, which occurs on steep, mesic, species-rich, W-SW-exposed slopes between 1300 and 1500 m a.s.l. (Béguin, 1972). Also interesting to note is the occurrence of *C.* thyrsoides* within the pioneer community Epilobietum fleischeri on the foreland of the Swiss Rhône-glacier on mostly siliceous ground (Schubiger-Bossard, 1988). This very site, however, is in close vicinity to a ruderalized population of *C.* thyrsoides* along the Furka Pass road and thus, the sub-spontaneous appearance within the Epilobietum fleischeri is most probably related to disturbances during the road improvements works conducted in the 1960's.

Populations of *C.* carniolica* are found in similar habitats as *C.* thyrsoides* and additionally within meso-thermophilic pine forests and juniper meadows (Jäger, 2000; Aeschimann et al., 2005). However, little phytosociological literature was available that mentioned *C.* carniolica* or could be referred to this subspecies unambiguously (Table 2). Further field surveys are therefore desirable.

Responses to abiotic factors

No information about water-, carbon-, and nutrient cycle is available. Also, experimental studies to understand the influence of light, temperature, soil pH, and nutrient availability for plant productivity and the species' resistance to frost, draught and/or heat stress is missing. An ecophysiological study may give important insights with respect to range and altitudinal limits of *C. thyrsoides*. For details about increased age at flowering with increasing altitude see the Reproduction chapter.

Abundance

In most populations *C.* thyrsoides* is scarce and reaches a coverage of only 1–5 %. Within permanent plots of 1 × 1-m abundance of vegetative rosettes was generally between 0 and 20 individuals. On average, 5 % of a population flowers and sets seed in a given season. However, there is considerable interannual fluctuation of flowering individuals between 0 and 10 % which is largely explained by demographic stochasticity (see Life-Cycle) and seasonal climatic fluctuation. A special situation is encountered on ruderal sites, such as abandoned construction sites or road shoulders. In the latter case, *C.* thyrsoides* can dominate the plant community and more than 200 vegetative rosettes and seedlings per m^2 have been found. Locally, populations follow mountain roads for several kilometers, being confined to the

carbonate-bearing road construction material in an otherwise siliceous bedrock environment (e.g. roads to Lac de Moiry and Furka Pass, Switzerland).

Life cycle and biology

Life cycle

C. thyrsoides is a rosette-forming monocarpic hemicryptophyte lacking means of vegetative propagation. Detailed investigations of the demography of *C.* thyrsoides* have been conducted at two sites in the Swiss Alps (Kuss, in prep.). Seeds germinate directly after snow melt, depending on altitude and snow cover, between May and July, with low establishment rates (see Germination). Seedlings have a high survival probability between 75% and 95%. Once grown into a vegetative rosette, survival rates of individuals increase to 90%-95%. Spring rosette development (see Morphology) is initiated right after snowmelt, the summer rosette at the peak of the vegetation period, usually in August. Vegetative rosettes steadily grow in size and successively increase the number of leaves. Growth rate was not density-dependent (R^2 = 0.001, P = 0.08). A general trend was detected toward decreased growth with increasing plant size, which is characteristic for most monocarpic species (Metcalf et al., 2003). The probability of flowering was strongly size-dependent (measured as the rosette size the previous year) with an altitude-independent flowering threshold of 20-30 leaves. If sister-rosettes are present they are autonomous and must each reach this threshold in order to flower. Flowering buds are initiated in the year of flowering shortly after snow melt and weak evidence for an additional age-dependent genetic regulation of flowering exists. In a common garden experiment at two different altitudes (Basel 290 m a.s.l. and Furka Pass 2450 m a.s.l., Switzerland) individuals of the same seed family tended to flower synchronized at both sites. Mean age at flowering in natural sites is 8 years with considerable variability (see Reproduction). Flowering commonly occurs between June-September (*C.* thyrsoides*) or June-August (*C.* carniolica*). Seed maturation requires approximately 3-4 weeks and seed dispersal starts in late August to September and can occur over the entire winter (see Reproduction). At high altitudes, cold years with a short vegetation period have been observed to lead to reproductive failure. Flowering plants will die at the end of the vegetation period, their vegetative sister rosettes will die, too.

Demographic analysis of two *C.* thyrsoides* populations within stable alpine grassland communities revealed that populations can moderately increase even at very low

establishment rates ($\lambda \approx 1.08$, Furka: $p_e = 0.016\%$, Schynige Platte: $p_e = 0.078\%$; Kuss, in prep.). Further elasticity analysis of the life-stage transitions using Integral Projection Models showed that survival and growth are the critical determinants of the population growth rate, λ, contributing 88-90% to the overall elasticities. Reproduction contributed only 10-12%. Additionally, the observed flowering strategy (size at flowering) was close to the predicted evolutionary stable strategy (ESS) indicating only weak selection for larger sizes at flowering.

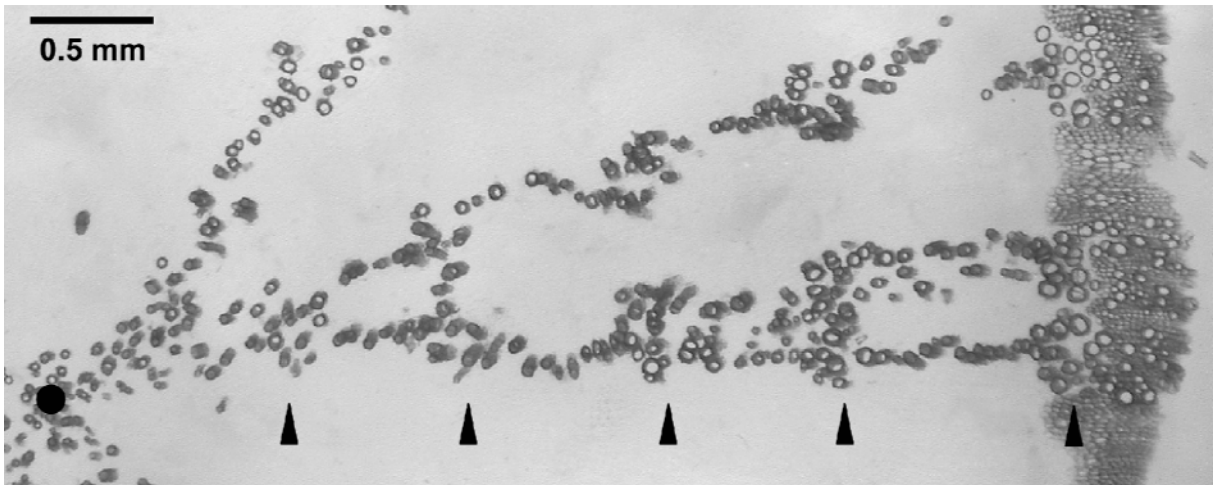

Figure 6. *Campanula thyrsoides*. Root cross-section of 5-year old vegetative plant. Dot: root center; arrows: wide-luminar spring cells.

Spatial distribution of plants within populations

See Abundance and Germination

Reproduction

Campanula thyrsoides is a monocarpic perennial, which flowers once and then dies. Traditionally, the species has been considered to be a „biennial“ plant (e.g. Murr, 1923-1926; Hayek, 1928-1931; Hegi, 1975) and only Jäger (2000) estimated flowering plant ages to range between 5-10 years, deducing from leaf numbers of large rosettes and annual leaf number increase. A recent survey of plant ages for *C.* thyrsoides* along an altitudinal gradient from 1450 m to 2430 m a.s.l. in Switzerland using ‘herb chronology’ (Dietz and Ullmann, 1997) dating techniques (Figure 6) revealed a mean flowering age of 7.5 years and a high variability ranging from 3-16 yrs ($n = 250$; Hänger, unpublished). Flowering ages significantly increased

with altitude (mean ages – Col du Marchairuz ,1450 m: 6.5 years, Schynige Platte, 1950 m: 7.8 years, Furka Pass, 2430 m: 8.3 years), such that the oldest flowering individuals were encountered at Furka Pass. Plants which flower in the second year were never encountered in the field and are also not very common (17%, $n = 451$) if grown under horticultural conditions in the lowlands.

C. thyrsoides has hermaphroditic strongly protandrous flowers. During bud stage, the anthers form a tube around the style, open introversely, such that pollen is deposited on the pollen-collecting hairs along the middle part of the style which then leads to secondary pollen presentation (Fægri and van der Pijl, 1979). During middle anthesis, late in the male phase, the three stigmatic lobes split open and the stigma becomes receptive. At that time, in a number of *Campanula* species, all pollen collecting hairs (PCHs) are retracted into the style starting at the uppermost part of the style and continuing downwards (Nyman, 1993a,b). The retraction of individual hairs starts at the base of the hair (comparable with the retraction of fingers of a glove) and is thought to be initiated by pollinator activity stimulating the PCHs. Late in anthesis, the stigmatic lobes bent backwards and can come in contact with the style. By then, all autologous pollen has usually either been collected by pollinators, fallen to the bottom of the flower or is hidden within the cavities of the retracted hairs such that self-pollination is avoided. However, in *C. thyrsoides*, PCH density is sparse and the retraction mechanism seems to be only imperfectly developed such that a sheath of pollen often remains around the style. Nevertheless, self-pollination is largely avoided because the uppermost part of the style is normally devoid of pollen so that the stigmatic lobes do not come in contact with autologous pollen.

C. thyrsoides is insect-pollinated, mainly by bumble bees and some smaller hymenopterans. Pollen transfer distances measured at the Furka Pass using fluorescent dye showed a maximum pollen flow distance of 39 m indicating pollen-mediated gene-flow to be restricted within single populations (9 marked plants, > 2000 potential recipient plants within 500 m, 1102 positive records at 173 plants. For detailed method see Stockhouse, 1976). Apomixis, vivipary or cleistogamy has not been observed. *C.* thyrsoides* is an obligatory outcrosser which barely sets viable seeds when experimentally selfed (Ægisdóttir et al., in press). *C.* thyrsoides* can be considered as semi-compatible, gametophytically self-incompatible, since it sets equally many seeds in outcrossed and sister-crossed flowers. In addition, the self-compatibility index (SCI) has been found to be very low for both young flowers (spontaneous and hand-selfing: 0.021) and old flowers (late selfing: 0.052) showing no breakdown of self-incompatibility with flower age (Ægisdóttir et al., in press). Breakdown

of the SI-system is however known in other *Campanula* species (Richardson et al., 1990; Vogler et al., 1998). All mentioned pollination experiments revealed no pollen limitation of reproduction. Furthermore, hardly any indication of inbreeding depression was found for sister-crossed offsprings (fitness measures: seed set, seed weight, germination percentage, survival probability, size of offsprings; (Ægisdóttir et al., in press). Under natural conditions self-pollination is usually avoided due to pollen removal by insects and further because little pollen is present where the stigmatic lobes may touch the style. Additionally, the probability of sister-mating is low because only 10-20% of a cohort flower in the same season and individuals of a cohort most likely come from different mothers (Hänger, unpublished). Despite this, weak evidence of inbreeding depression exists when seed set from close neighbour crosses are compared to distant neighbour crosses (Ægisdóttir et al., in prep.).

In approximately 10 % of the plants, one or rarely up to six sister rosettes are formed, which are autonomous with respect to flowering initiation (Figure 2 h). However, vegetative reproduction is not possible because all sister rosettes are connected to the main tap root from which the mobile carbon reserves will be exclusively invested in any flowering rosette.

Seed set occurs in 90-100 % of all flowers under natural conditions. The number of flowers and thus, the number of seeds per plant is strongly dependent on the size of the rosette measured the previous year (Kuss, in prep.). In *C.* thyrsoides*, an average plant had 60 flowers (± 34 SD, min: 28, max: 156, n = 122), with an average number of well-developed seeds per capsule of 147 (± 91 SD, min: 0, max: 633, n = 500). The number of seeds per capsule has been found to be size- and site-dependent with greatest seed production per capsule at a high altitude site at Furka Pass (Ægisdóttir et al., in prep.). Overall fecundity per plant is, again, strongly size-dependent and ranges between 15000 and 50000 viable seeds (n = 122; Kuss, in prep.). Seed/ovule ratio was 0.65 (± 0.13 SD, n = 500). Well-developed viable seeds weigh on average 0.11µg (± 0.03 SD, n = 100) and measure 1.05mm (± 0.15 SD, n = 100). The margin is only rudimentarily winged and thus, seeds lack special adaptations to dispersal. Primary dispersal is by wind, rain or animals shaking the seeds out of the withered infructescence (see Morphology) but detailed studies are missing. Long-distance dispersal has been modelled using a high-alpine wind data set implemented in the software PAPPUS (Tackenberg, 2003) with a fixed terminal falling velocity V_{term} = 1.36 ms^{-1} and a release mean height H_{rel} = 0.25 m. About 99.99% of the seeds are dispersed within <10 m of the mother plant, and only 0.001% > 100 m and 0.001% >1000 m. With an average seed production of 1.5 million seeds per populations this implies that approximately 15 seeds are potentially

dispersed over 1 km. Secondary anemochory over snow and ice is well probable after the release of seeds out of the lignified infructescence during winter time.

Germination

Seed germination is epigeal and occurs directly after snowmelt between May and July, depending on altitude and snow cover. Seedlings have a shortened epicotyl and condensed basal internodes (Figure 3). The cotyledons are oval, 4-6 mm long and 4-5 mm broad, with unicellular hairs mostly along the margin and infrequently on the surface of the cotyledon. In permanent plots we found an average germination rate of 0.016% (Furka Pass) and 0.078% (Schynige Platte). Despite seed production per 1 × 1 m quadrat varying from 0 to 68 000 seeds, the maximum number of seedlings recorded the following year was 25. At both sites there was no relationship between per quadrat seed production and subsequent recruitment. Further, we found no indication that seedling establishment was negatively density dependent. At Furka Pass, recruitment was independent of plant densities, while at Schynige Platte seedling establishment increased significantly with increasing plant densities (Kuss, in prep.). These and the following results indicate that microsite limitation is most influential on the establishment rate of seedlings. In a common garden experiment at Furka Pass with seeds from 10 populations of different altitudes, germination rates varied greatly between 0 and 3% with no observed effect of altitudinal origin of the population. Seeds collected one year prior to the experiment and stored at 4°C still germinated at similar rates. For the common garden study, all seeds were collected at the time of seed maturation in the fall and sown shortly afterward in cleaned autochthonous soil. Under laboratory conditions seed germination rates on wet filter paper reached 79% (Ægisdóttir et al., in press). In an additional experiment, application of varying levels of Giberrelic Acid (10-1000 mg/l) had no effect on germination success or timing of germination (Jespersen, 2005). A thorough soil seed bank experiment is still lacking but no literature or field indication exists for a persistent seed bank (Hegi, 1975).

Response to Competition and Management

C. thyrsoides seems to be adapted to a moderate disturbance regime and populations persist within extensively used meadows and pastures most probably for hundreds of years. Unfortunately, no long-term permanent plot data including *C. thyrsoides* exists for Switzerland (Krüsi and Vittoz, pers. comm.) and only indirect evidence is available from old

phytosociological relevés (e.g. Lüdi, 1948) that show almost no change in species' frequency/abundance compared to the current condition. There is also no indication that the traditional 2-year hay-making practice above the tree-line (German: *halbschürige Mahd*) of many Alpine cultures has diminished population numbers. However, recent intensification of grazing pressure by cows, and locally sheep, is reported to have reduced population numbers and sizes in the last decades (Seitter, 1989). In contrast, *C. thyrsoides* can been found on numerous road shoulders and construction sites where disturbance has stopped few decades ago. In such places, populations can rapidly expand and sometimes amount to several 100 000 individuals. *C. thyrsoides* usually occurs in naturally ahemerobe or oligohemerobe habitats such that little conservation measures have to be applied to assure long-term persistence (Hegg et al., 1993).

Herbivores and Pathogens

Alpine ungulates such as chamoix (*Rupicapra rupicapra*), roe deer (*Capreolus capreolus*), ibex (*Capra ibex*) or marmot (*Marmota marmota*) are natural herbivores in habitats of *Campanula thyrsoides* and browsed flowering plants and rosettes have been observed in locations where no livestock grazing or human activity takes place. Nevertheless, herbivory under natural conditions seems very low. On the other hand, plants from cow and sheep pastures were often found decapitated.

Observed pre-dispersal seed predators in *C.* thyrsoides* were a small leafroller moth, *Cochylis pallidana* Zeller (Tortricidae; Tortricinae), and two weevil species, *Miarus* cf. *graminis* (Gyll) and *M*. cf. *abeillei* (Desbrochers). Infection with *Cochylis* larvae and *Miarus* spp. were present in all 9 *C.* thyrsoides*' populations censused in Switzerland. About 40% (range 8-78%, 2005) and 83% (range 47-100%, 2006) of all plants were infected, and in infected plants 23% (range 0.007 - 93%, 2006) of capsules were predated and predation caused complete seed loss. Seed predation by *Cochylis* larvae was usually higher than by *Miarus* spp. (67% vs. 33%, respectively). In the literature *Cochylis pallidana* has been described to feed exclusively on *Jasione montana* (Razowski, 1970), a relative of *Campanula* that is wide-spread throughout Europe (Meusel and Jäger, 1992). It remains to elucidate whether the moths feeding on *C.* thyrsoides* belongs to a yet undescribed ecological race or subspecies of *Cochylis pallidana* as both host plants rarely occur in close vicinity. *Miarus graminis* has been recorded to feed on *Campanula glomerata* but little is known about *M. abeillie* (Stevenson et al., 1997).

Mycorrhiza

C. thyrsoides* is colonized by endomycorrhizal fungi with root colonization rates usually below 25 %. Only three fungal species could be isolated using spore trap cultures with soil collected in a typical habitat: *Glomus constrictum*, *G. invermaium* and *G. versiforum*. However, a larger spectrum of symbionts is most likely, given the range of natural habitats and the species' altitudinal distribution.

Physiological data

No data available for this species.

Biochemical data

No specific data on *C. thyrsoides* available. A detailed study on mobile carbohydrates, its storage and allocation during different life-stages is currently performed (Ch. Schädel, Uni Basel, Switzerland). The white latex present in all plant parts is most likely inulin, or fructan, as these substances are characteristic for Campanulaceae serving as long-term winter storage compounds in tap-roots and playing a functional role during floral development (for *C. rapunculoides* see Vergauwen et al., 2000).

Genetic data

C. thyrsoides is diploid, $2n = 34$ (Rosen, 1931; Larsen, 1954; Gadella, 1964), and this chromosome number is shared by about 42 % of the investigated members of the family Campanulaceae s.l. (Lammers, 1992). Additional chromosome counts by Sugiura (1942), with $2n = 48$, are considered incorrect and are commonly not taken into account for comparative studies (Gadella, 1964; Hess et al., 1972).

In a screening test with 18 allozyme systems on seedling tissue of *C.* thyrsoides*, no polymorphisms were detected (allozyme systems: AK, ADH, AO, ARK, AAT, G6PDH, GPI, HEX, IDH, LDH, MDH, NADP+(ME), PGM, 6PGDH, SOD, α-Trehalase, TPI, XDH; method: CAGE in TG buffer run for 15-30 min at 25mA; Ægisdóttir, unpublished).

A population genetic study on *C.* thyrsoides* of 32 populations in the Swiss Alps and Jura mountains using RAPD markers found moderately high within-population diversity values (Nei's genetic diversity, H_e: 0.20 (± 0.003 SE); Shannon Index, SI: 0.32 (± 0.006 SE); Percentage of polymorphic loci, P_p: 61.8 (± 1.3 SE); n = 32)(Kuss, in prep.). Within-population diversity was not influenced by altitudinal location or population size. Even small populations of <100 individuals had comparably high levels of genetic diversity and none of the studied populations seems to be at immediate risk of extinction as a result of genetic depauperation. Spatial analysis of population relatedness detected a sharp genetic contrast between populations from the western part of Switzerland and the central and eastern populations. This delineation coincides with the proposed border of two distinct areas of post-glacial migration which are separated by the north-south running Aosta-Rhône-Valley (Schönswetter et al. 2005) and is also visible in the geographic distribution map (Figure 4: line A). Morphological differences however could not be found between populations from the two distinct areas.

Among-population diversity within a single glacial refugium in Switzerland was relatively low, with a mean Φ_{st}-values of 16.8 % (± 0.003 SE) and G_{st}-value of 18.2 (± 0.03 SD). Pairwise Φ_{st}-values ranged from 2.3 % to 29.3 % and a significant isolation by distance behavior could be found (R^2 = 0.32, P = 0.007) indicating restricted gene flow. Despite the spatial isolation of populations, population differentiation was only moderately high and may be explained by the outcrossing breeding system of *C. thyrsoides*, which ensures successive gene pool mixing, and counteracts differentiation by drift.

A population genetic study with the same samples but applying microsatellites is currently in progress. Preliminary results confirm a relatively high within population genetic diversity as well as a significant isolation by distance pattern as found when using the RAPD markers (H.H. Ægisdóttir, Uni Basel, Switzerland).

Hybrids

Hybrids of *C. thyrsoides* have not been observed in nature. In the only known experimental hybridization between the closely related *C. spicata* × *C. thyrsoides*, seedlings died in a very early stage (Gadella, 1964).

Status of the species

C. thyrsoides is rare throughout its native range but locally abundant and therefore not considered endangered as a species. However, due to its regional rarity, *C. thyrsoides* is listed in a number of Red Lists from the different Alpine countries and categorized according to the IUCN-criteria (e.g. Moser et al., 2002): Austria – '*near threatened*' (Niklfeld and Schratt-Ehrendorfer, 1999), Bulgaria – '*endangered*' (Anchev, pers. comm.), Croatia – '*strictly protected*' (Nikolić, 2006), France – '*least concern*' (Ferrez, 2004), Germany – '*vulnerable*' (Korneck et al., 1996), Switzerland – '*least concern*', but regionally '*vulnerable*' or '*near threatend*' (Moser et al., 2002). The species is not mentioned in the 'Red Lists' of Italy (Pignatti et al., 2001) and Slovenia (Wraber, pers. comm.). Information from Bosnia-Herzegovina were not available.

C. thyrsoides is protected at the national level in Germany (Bundeamt für Naturschutz, 2006). In Austria and Switzerland the species is protected at some regional but not national level (Switzerland: Appenzell-Innerrhoden, Graubünden, Nidwalden, Obwalden, St. Gallen, Ticino; Vust and Galland, 2001; and Austria: only *C.* carniolica* in Kärnten; Kiss et al., 2006). The species is not included in the Natura 2000 (Council of Europe, 1992), CITES (IUCN, 2004) or Bern Convention treaties (Council of Europe, 2006).

C. thyrsoides has little commercial value, is rarely grown or sold as an ornamental plant and has no known medicinal use.

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Chapter 9

Summary and general discussion

This thesis focuses on the breeding system and the reproductive ecology (Chapter 2-3) as well as on the genetic diversity and geographic structure (using microsatellites markers, Chapter 4-5) within and among populations of the rare monocarpic perennial, *Campanula thyrsoides* in the naturally fragmented alpine landscape. Furthermore, this thesis includes three additional chapters, of which I am a co-author. These chapters are firstly a genetic study on three alpine plant species using a dominant marker (RAPD) (Chapter 6), a study on the evolutionary demography (Chapter 7) and a monography on all available information on *C. thyrsoides* (Chapter 8).

Plant reproduction and genetic variation within and among plant populations can be affected by several factors, such as life history traits and habitats. Plants of alpine habitats are considered to be affected by the strong natural fragmentation, produced by pronounced environmental gradients and heterogenous topography that often characterizes these habitats (Körner 2003). The geographical barriers of alpine landscapes demarcate the habitat ranges of the plants, which can hinder seed dispersal, a prerequisite in order for plants to be able to colonize new sites (Cain et al. 2000; Theurillat & Guisan 2001). Such restricted gene flow should lower genetic diversity within alpine plant populations and increase genetic differentiation among populations (Till-Bottraud & Gaudeul 2001). Moreover, self-compatibility should be favoured in plant species living in isolated populations in a fragmented landscape in order to ensure sexual reproduction if pollination fails (Baker 1955, 1967).

Genetic diversity and differentation in plants can also be influenced by other factors, such as population size and reproductive system. Genetic drift can be enhanced in small populations, which could lead to extinction of alleles and loss of genetic variability (Barret & Kohn 1991; Ellstrand & Elam 1993). The reproductive system of plants can have a major impact on genetic diversity, with outcrossing species frequently having high within-population genetic diversity and low genetic differentiation, whereas mainly selfing species more commonly have low variability within populations and high differentiation between populations (Hamrick & Godt 1996; Booy et al. 2000).

To study the effects of natural fragmentation and patchiness of alpine habitats on *Campanula thyrsoides,* we firstly set up an experiment in the common garden in order to explore the breeding system and the consequences of selfing, half-sibling mating and outcrossing on reproductive output and seedling performance (Chapter 2).

Additionally, we studied the reproductive ecology in field populations in the Swiss Alps, where we examined whether pollination distances affect reproductive output within and between populations of *C. thyrsoides*. In these studes, we especially asked if we find a distance related inbreeding depression within populations and a hidden inbreeding depression or outbreeding depression in flowers following large-distance compared to within-population crossings (Chapter 3). In Chapter 5, we report on our study on the genetic diversity, gene flow and geographic structure within and among 32 population of *C. thyrsoides* throughout the Swiss Alps using co-dominant microsatellite markers (see development of microsatellite markers in Chapter 4). The genetic variation of *C. thyrsoides* was, furthermore, studied with the dominant marker Random Amplified Polymorphic DNA (RAPD) and the results compared with two other Alpine plant species; *Epilobium fleischeri* and *Geum reptans* (Chapter 6). In order to understand the mechanism behind delayed flowering in the monocarpic *C. thyrsoides*, we studied the evolutionary demography of the species by combining permanent plots and herb chronology data from two study sites in the Swiss Alps (Chapter 7). The last chapter (Chapter 8), reviews all data that are available on *C. thyrsoides*, from the taxonomy and habitat of the species, through to reproduction and the genetic and conservation status of the species within its native range.

Breeding system and consequences of selfing and half-sibling mating in the common garden

To study the breeding system of *C. thyrsoides* and to explore if the species has evolved towards self-compatibility in the alpine landscape we set up a pollination experiment in the common garden. We tested the effects of selfing (sponteneous selfing, hand-selfing, late selfing), half-sibling crossing, outcrossing and controls on reproductive output and seedling performance (Chapter 2).

Our results indicate that *Campanula thyrsoides* has a strong self-incompatibility system since it set no or very few seeds when selfed (with an average of five seeds per capsule). Unlike some other *Campanula* species (e.g. *C. rapunculoides*, see Vogler et al. 1998), the self-incompatibility system did not break down with flower age in *C. thyrsoides*. Seed set was as prominent in outcrossed as in sister-crossed flowers, indicating that the species has a gametophytic self-

incompatibility system and therefore has the same floral development as most other *Campanula* species (Stephensen et al. 1992; Richards 1997). The self-compatibility index was very low, indicating that the self-incompatible system was stronger than we expected for a species living in the fragmented alpine landscape where self-compatibility should be favoured (Baker 1955, 1967). Surprisingly, there was no significant difference in seed set, seed weight and germination percentage between outcrossed and sister-crossed flowers. Equally, no difference was detected in the survival and size of outcrossed and sister-crossed offspring. These results suggest that no inbreeding depression is found in the species, which could be explained by frequent bottlenecks during colonization of isolated habitat in the fragmented alpine landscape.

Pollination-distance effect on reproduction: field studies

We examined whether pollination distances affect reproductive output within (self, 1m 10m and 100m) and between populations (distances between populations: 3-113 km) of *C. thyrsoides* field populations in the Swiss Alps. We firstly asked if the self-incompatibility system of *C. thyrsoides* is strictly fixed, secondly, if we would find a distance related inbreeding depression within *C. thyrsoides* populations, and lastly if we find evidence of hidden inbreeding depression or outbreeding depression in flowers following large-distance crossings compared to within-population crossings. We sought answers to these questions by setting up two separate field experiments over two field seasons in Switzerland. To test for consequences of pollination distances within *C. thyrsoides* populations, we set up a crossing experiment in four field populations whereas two of these four populations were used as sites for the between-population crossing experiment (Chapter 3).

Although both spontaneously selfed and hand-selfed flowers set much fewer seeds compared to control and outcrossing flowers, enough seed set was present to conclude that the self-incompatible system in *C. thyrsoides* is not strictly fixed. A certain degree of variation or plasticity in the self-incompatible system was to be expected for a monocarpic species living in a naturally fragmented landscape (Baker 1955, 1967; Ghazoul 2005). All four field populations reacted similarly to pollination distances within populations, with flowers pollinated with pollen 10m away from the target plant outperforming the shorter distance (1m away), indicating an inbreeding

depression in the 1m treatment plants. Since, seed and pollen dispersal is limited in *C. thyrsoides* (see Chapter 5, Chapter 8), we expect neighbouring plants to be more genetically related than plants further away (Turner et al. 1982; Sokal & Wartenberg 1983). This suggests that the lower seed set in 1m distance is due to mating between closely related individuals. Our results, moreover, indicate that an optimal outcrossing disance exists within the *C. thyrsoides* populations, defined as the distance where seed set reduction due to inbreeding- and outcrossing depression is not present. Several studies have reported similar outperformance of intermediate crossing distances (3-10m) in plant populations compared to shorter (self, 1m) and longer (100m, 1000m) distances (e.g. Price & Waser 1979; Waser & Price 1994). Crossings between populations resulted neither in an increased nor a decreased seed set and seed germination, indicating no hidden inbreeding depression or outbreeding depression in our studied *C. thyrsoides* populations.

Genetic diversity and gene flow in a naturally fragmented landscape

The genetic diversity, gene flow and geographic structure within and among 32 populations of *C. thyrsoides* was studied throughout the Swiss Alps. We used firstly microsatellite co-dominant markers (Chapter 5), which were developed and characterized for our study species (see Chapter 4). Secondly *C. thyrsoides* was examined with the dominant marker Random Amplified Polymorphic DNA (RAPD) (Chapter 6).

By using 5 microsatellite loci, we found a high level of mean genetic diversity within populations (expected heterozygosity; $H_E = 0.76$), which is a higher value than many other microsatellite-based studies on plants (Nybom 2004). Moreover, we found a high outcrossing rate (average: 0.96) and low inbreeding coeffiecient ($F_{IS} = 0.022$) in our studied populations. By using RAPD, we found moderately high within-population genetic diversity (Nei's genetic diversity, $H_E = 0.20$; Shannon Index = 0.32; percentage of polymorphic loci, Pp = 61.8). Since plants' breeding systems are believed to have a significant influence on the distribution and magnitude of genetic diversity, with outcrossing plant species generally having higher genetic variation than mainly selfing species (Loveless & Hamrick 1984; Hamrick & Godt 1996), we

suggest that the breeding system of *C. thyrsoides* (outcrossing, largely self-incompatible) drives the high within-population diversity of the species.

Despite an existing theory's predition that small populations lose genetic variation because of e.g. population bottlenecks and genetic drift (Ellstrand & Elam 1993; Young et al. 1996), population size did not have any effect on molecular diversity in *C. thyrsoides* by using microsatellites and RAPD markers. The genetic diversity in other alpine species, e.g. *Geum reptans* (Pluess & Stöcklin 2004) and *Saxifraga oppositifolia* (Gugerli et al. 1999) have also been reported to be independent of population size. Despite the fact that plants are often considered to be exposed to stronger selection at higher altitude due to the harsh alpine environments, which could lead to lower genetic diversity within plants populations in alpine environments compared to populations at lower altitudes (Till-Bottraud & Gaudeul 2002), we did not find evidence suggesting a lowering of genetic diversity with increasing altitude (with both marker systems). That is in agreement with other studies on alpine plants (e.g. Gugerli et al. 1999; Pluess & Stöcklin 2004; Till-Bottraud & Gaudeul 2002).

By using microsatellites, the inbreeding coefficient (F_{IS}) was reported low in all 32 populations, although most population values were positive. Hence, 11 populations of 32 deviated significantly from Hardy-Weinberg equilibrium (HWE). We find inbreeding due to half-sister mating, the most likely explanation for the deviance from random mating (HWE) in *C. thyrsoides*.

By using microsatellites, we observed a moderate genetic differentiation among the *C. thyrsoides* populations (F_{ST} = 0.103) and a significant and positive isolation-by-distance relationship for both microsatellites (R^2 = 0.39, $p < 0.001$) and RAPD (R^2 = 0.53, $p < 0.001$), which indicates a restricted gene flow between populations as would be expected for a species living in the fragmented Alpine landscape (Cain et al. 2000; Theurillat & Guisan 2001). A restricted gene flow was, moreover, indicated by the detection of first generation migrants which showed that only 18 individuals had a probability value below 0.01 and were therefore considered migrants. The overall genetic differentiation was, in spite of this, not particulary high, when the highly limiting dispersal capacity and the life-history of the species (being monocarpic, which should promote genetic differentiation; Loveless & Hamrick 1984) is taken into account. By using STRUCTURE for analysing our data, we found a clear geographical boundary existing between populations in Western Switzerland

and populations in Central-Eastern Switzerland. Since the observed West-East division in our populations did not correspond to the geographical division between the Jura mountains in the West and the Alps in the East, we consider it most likely that this genetic boundary arose as a result of post glacial colonization history, as it corresponds to the glacial refugia map for mountain plants in the European Alps (see Schönswetter et al. 2005). By using RAPD, we also detected a sharp genetic contrast between populations in West Switzerland and Central-Eastern Switzerland.

Evolutionary demography of *Campanula thyrsoides*

Lastly, the evolutionary demography of *Campanula thyrsoides* was studied by sampling data from permanent plots and herb chronology to use in a time-lagged integral projection model (IPM). The model was mainly used to predict the age-structure observed in the field and to determine the evolutionary stable flowering strategy (Chapter 8). The most important findings of this study were that like in many other monocarpic perennials (Metcalf et al. 2003), all basic demographic functions (growth, survival probability, flowering probability, and fecundity) were strongly dependent on plant size. There was also a prononunced threshold size of flowering which is in accordance to other monocarpic perennials and may be explained by the accumulation of a minimum amount of stored reserves required for flowering (Young & Augspurger 1991).

Summary & main conclusions

The results presented in this thesis, indicate that the outbreeding habit of *Campanula thyrsoides* plays a central role in maintaining a high genetic diversity within its populations. The outbreeding habit is, moreover, likely to buffer possibly negative effects of high altitudes and small population sizes on molecular diversity in *C. thyrsoides*.

Although, the breeding system of *C. thyrsoides* has not evolved towards complete self-compatibility, its self-incompatibility system is not strictly fixed as was to be expected for a monocarpic species living in isolated populations. We detected a inbreeding depression after crosses between close neigbour plants and a deviance

from random mating (Hardy Weinberg equilibrium) in 11 out of 32 studied populations. This indicates that the outbreeding habit of *C. thyrsoides* is likely to be maintained with inbreeding depression. Negative effects of detrimental mutation accumulation (which are likely to cause the inbreeding depression in *C. thyrsoides*) are expected to increase with an increasing duration of population isolation and smaller size of population (Lande, 1995). Given this, inbreeding depression could become disadvantageous for outcrossing species (like *C. thyrsoides*) that live in isolated habitats in the alpine landscape, if populations are very small. This may explain why only sparsely distributed *C. thyrsoides* populations occur in the Alps.

Despite that the fragmented landscape of the Alps clearly restricts gene flow among our study populations, indicated by a significant isolation by distance relationship and a moderate genetic differentiation, we observed lower genetic differentiation than would be expected in a monocarpic perennial that lives in the fragmented alpine landscape and, moreover, has a restricted seed dispersal capacity. We, furthermore, found a clear geographical boundary among populations in Western versus Central/Eastern regions of Switzerland, which could be related to post glacial colonization history. This suggests either a greater recent gene flow between populations than expected, or a historical gene flow which has had insufficient time to allow genetic differentiation to occur (e.g.: since the last glacial period).

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1997-2000	BSc in Biology, University of Iceland, Reykjavík, Iceland. Research Project: Seed production and seed quality of *Saxifraga hirculus* in a large, continuous habitat compared to small, isolated habitats (in Icelandic).
2000-2003	MSc in Biology, University of Iceland, Reykjavík, Iceland. M.Sc. thesis**:** Reproductive ecology, morphological and genetic diversity in *Campanula uniflora* in Iceland, Greenland and Svalbard. Supervisor: Prof. Thóra Ellen Thórhallsdóttir. From 09/2000 – 06/2002. Guest student at the Botanical Institute, University of Copenhagen, Denmark.
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Work Experience

Summer 1998	Public Health Authority of the Hafnarfjördur Area. Responsibilities: study on the water protectorate of the towns of Hafnarfjördur and Garðabær, Iceland
Summer 1999	Research assistance in Thjórsárver Nature Reserve, central Iceland.

Summer 2002	Core teacher in the summer program "Arctic Biology", in Iceland (Denmarks International Study Program in partnership with the University of Iceland).
2001-2003	Teaching assistance in several courses in the Faculty of Natural Sciences. University of Iceland.
Summer 2005	Leading and organizing 11 day ecological field excursion to Iceland for MSc and PhD students from the University of Basel.

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