No inbreeding depression in an outcrossing alpine species: The breeding system of *Campanula thyrsoides*

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**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 short-lived 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.

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**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; Karren, 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 favored 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 *Campanula uniflora* (Egisdóttir and Ólafsson, 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 rapunculoides*, 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 (Egisdóttir and Ólafsson, 2006; Ólafsson and van der Pijl, 1979).

Our study species, *Campanula thyrsoides*, is a short-lived monocarpic perennial, living at high altitudes in the Alps, frequently in isolated habitats of calcareous soils. Although most *Campanula* species are SI 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 is not, 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* L. 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 inflorescence 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).

*C. 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).

**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 four treatments and three replicates by randomly choosing flowers of a single plant, i.e. we applied different treatments 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 three 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 20 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 six seed families comprising three 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 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: \( p = 0.07, 0.13 \)).

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 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 ($p = 0.91$).

**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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (mg)</th>
<th>Seed germination (%)</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.17 (+2.42)</td>
<td>78.80 (+3.93)</td>
<td>7</td>
</tr>
<tr>
<td>Outcrossing</td>
<td>13.71 (+1.59)</td>
<td>88.65 (+2.85)</td>
<td>26</td>
</tr>
<tr>
<td>Sister crossing</td>
<td>15.90 (+1.84)</td>
<td>82.73 (+3.85)</td>
<td>26</td>
</tr>
<tr>
<td>Hand-selfing</td>
<td>0.09 (+0.04)</td>
<td>—</td>
<td>26</td>
</tr>
<tr>
<td>Spontaneous selfing</td>
<td>0.14 (+0.07)</td>
<td>—</td>
<td>26</td>
</tr>
<tr>
<td>Late selfing</td>
<td>0.17 (+0.08)</td>
<td>—</td>
<td>26</td>
</tr>
</tbody>
</table>

$N =$ number of plants per treatment.

<table>
<thead>
<tr>
<th>Rosette diameter 15 weeks</th>
<th>Rosette diameter 25 weeks</th>
<th>$N$ leaves/rosette 25 weeks</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63 (+3.17)</td>
<td>176 (+4.62)</td>
<td>55 (+1.82)</td>
</tr>
<tr>
<td>Outcrossing</td>
<td>58 (+6.61)</td>
<td>191 (+4.22)</td>
<td>52 (+2.06)</td>
</tr>
<tr>
<td>Sister crossing</td>
<td>55 (+5.42)</td>
<td>181 (+4.60)</td>
<td>50 (+1.64)</td>
</tr>
<tr>
<td>Selfing</td>
<td>65 (+4.4)</td>
<td>186 (+7.55)</td>
<td>53 (+2.37)</td>
</tr>
</tbody>
</table>

$N =$ number of seedlings per treatment.
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 it 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 Hol singer, 2002; Stephenson et al., 1992).

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 an SCI of less than 0.15 are defined as strong SI plants while plants with an 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 SI 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 a 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 (Glémín 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 situation 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 cannot 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 SI. 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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