

Research Article

Better than nothing: Evolution of autonomous selfing under strong inbreeding depression in an alpine annual from the Qinghai–Tibet Plateau

1,2Chan ZHANG 3Guo-Ying ZHOU 1,4,5Yong-Ping YANG 1,4,5Yuan-Wen DUAN*

¹(Key Laboratory for Plant Biodiversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China)²(University of Chinese Academy of Sciences, Beijing 100049, China)³(Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China)⁴(Plant Germplasm and Genomics Center, The Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China)⁵(Institute of Tibetan Plateau Research at Kunming, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China)

Abstract In plants with infrequent pollinator services, the benefits of reproductive assurance could be eroded by severe ovule discounting and inbreeding depression (ID). However, it remains unclear how selfing evolves under complete pollinator failure and strong ID. We examined the mating system and ID under netting and robbing conditions in *Comastoma pulmonarium* (Turcz.) Toyok. (Gentianaceae), an alpine annual experiencing a high ratio of nectar robbery on the Qinghai–Tibet Plateau. *Comastoma pulmonarium* produced seeds via selfing at the study site. No pollinator was observed and thus the nectar was consumed exclusively by robbers. Inbreeding depression occurred in the life stages of seed mass and germination, and the cumulative ID was much higher than 0.5 under netting and robbing conditions. Overall, in comparison with netting, the magnitude of ID under robbing conditions tended to decrease. Our results suggested that selfing could assure reproduction for plants under complete pollinator failure and strong ID, supporting the “better than nothing” role of selfing and providing one of the few cases of the evolution of selfing under strong ID.

Key words autonomous selfing, *Comastoma pulmonarium*, Gentianaceae, inbreeding depression, nectar robbery, pollinator failure, reproductive assurance.

Evolution towards selfing has been considered as one of the most common evolutionary trends in angiosperms (Stebbins, 1974), and theoretical models suggest two opposing consequences of this evolutionary trend. Selfers provide a 50% gene transmission advantage over outcrossers (Fisher, 1941). In contrast, self-pollination is detrimental when selfed offspring suffer reduced viability due to inbreeding depression (ID) (Charlesworth & Charlesworth, 1987). Therefore, the mating system should evolve towards either predominant outcrossing when ID exceeds 0.5, or predominant selfing when ID is less than 0.5 (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987; Jarne & Charlesworth, 1993).

However, mixed mating systems occur widely in plants (Goodwillie et al., 2005), indicating that we cannot consider the gene transmission advantage and ID alone (Vaughton et al., 2008). Reproductive assurance,

when pollinators and/or potential mates are scarce, is the most longstanding and widely accepted explanation for the evolution of selfing (Kalisz et al., 2004 and references therein; Zhang & Li, 2008; Duan et al., 2010). Nevertheless, the increase in seed production due to reproductive assurance through autonomous selfing might be outweighed by severe ovule discounting and ID, challenging the widely held explanation for the evolution of selfing (Herlihy & Eckert, 2002; Vaughton et al., 2008). Moreover, if pollinators are absent completely, then no pollen and/or ovule discounting occur (Holsinger, 1991; Lloyd, 1992), and thus ID might act as the only factor preventing the evolution of selfing. However, it remains unclear how selfing evolves under strong ID and whether there are some mechanisms used by plants to reduce the magnitude of strong ID in selfing plants.

In *Comastoma pulmonarium* (Turcz.) Toyok. (Gentianaceae), we have found strong ID in the early life history and selective seed abortion induced by nectar robbery (Zhang et al., 2011), but it is still unknown how many seeds were produced via outcrossing and what

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* Author for correspondence. E-mail: duanyw@mail.kib.ac.cn. Tel./ Fax: 86-871-65223231.

was the magnitude of ID in the late life history after selective seed abortion. Here, we present our results on the breeding system and ID of *C. pulmonarium*. Specifically, our two objectives were to test the dominance of selfing in *C. pulmonarium*, and to examine the difference in the magnitude of ID in progenies from netted and robbed flowers in the late life history.

1 Material and methods

1.1 Study site and species

Our studies were carried out around the Haibei Alpine Meadow Ecosystem Research Station (37°29′–37°45′ N, 101°12′–101°23′ E, 3200 m a.s.l.) (Chinese Academy of Sciences, Qinghai Province, China) from July to September in 2001, 2009, and 2010. We carried out experiments and observations in one population in 2001 and 2010, and in three populations in 2009.

Comastoma pulmonarium is an alpine annual herb inhabiting altitudes ranging from 3000 to 4800 m a.s.l. The plant is 5–30 cm in height and flowers in July and August. The corolla of tubular flower is pale blue, with five corolla lobes. There are two whorls of fringed white fimbriae at the base of elliptic lobes, covering anthers and stigma completely. Ten nectaries are situated at the base of corolla tube. Fruits become mature from August to September.

1.2 Self-compatibility

To examine the self-compatibility of *C. pulmonarium*, we netted two groups of flower buds on different individuals, and emasculated them before anther dehiscence in 2001 and 2009. After stigma opened, one group of flowers were hand selfed using pollen grains from the same plant, and the other group of flowers were hand outcrossed using pollen grains from different plants approximately 10 m away from the recipient plants. Pollen grains used in hand pollination were from recently dehisced anthers to ensure the same pollen age, and we collected the two groups of flowers 2 weeks after hand pollination to exclude the effect of early acting ID on the seed-set ratio. We counted the numbers of developing ovules and non-developing ovules (including unfertilized ovules and aborted ovules) to calculate the seed-set ratio, which was compared between the two groups using the independent samples *t*-test.

1.3 Contributions of self-pollen and alien pollen to seed-set ratio

Three groups of buds on different plants were selected in one population in 2001 and in each of the

three populations in 2009, and subjected to the following treatments: (i) emasculation and open pollination; (ii) netting without emasculation; and (iii) open pollination (control). We also collected the three groups of flowers 2 weeks after commencement of the experiment to determine the seed-set ratio, which was compared using a three-way ANOVA with treatment as the fixed factor and year and population as random factors.

1.4 Observations of visitors

The floral visitors of *C. pulmonarium* were observed in one population in 2001 and in three populations in 2009. Each day before observations, 20 neighboring open flowers on different individuals were labeled. We observed these flowers from 09:00 to 17:00. In total, we carried out observations on 80 flowers in 20 h in 2001 and 300 flowers in 60 h in 2009. During these observations, we stayed 2 m away from the focal flowers to observe all the floral visitors without disturbing their foraging behaviors, and the weather was fine without strong wind.

1.5 Nectar production

In the three populations in 2009, we randomly bagged some flowers at 08:00, and checked the nectar volume using microcapillaries and Vernier calipers on the next day at 08:00.

1.6 Inbreeding depression

In 2010, we selected 120 flowers on different plants and separated them into two groups (60 flowers for each group). For one group, all flowers were emasculated before anther dehiscence and netted to exclude robbers. After stigma opened, 30 flowers were subjected to hand selfing using pollen grains from the same plant, and the other 30 flowers were subjected to hand outcrossing with pollen grains from different plants 10 m away from the recipient plants. For the other group, all flowers were open to robbers after emasculation in bud stage. In addition, 30 flowers were hand selfed and another 30 flowers were hand outcrossed in this group. Only the terminal flowers were used for this hand pollination experiment to avoid the effect of position on the ovule number on one plant. We collected the mature fruits just before dehiscence to determine the number of mature seeds of each treatment. In addition, for the group exposed to robbers, if we found no hole on the corolla when collecting fruits, we would give up this fruit. These methods were the same experiments from our former studies (Zhang et al., 2011).

In the laboratory, after being air dried, seed mass of 100 seeds from each treatment was weighed for 12 replications using an electronic balance (0.1 mg). Then all seeds were kept at -20°C for 1 month to break dormancy, and three replicates of seeds (34–40 seeds for each replicate) from each treatment were placed in small Petri dishes with wet filter paper. Three small Petri dishes were put in a larger Petri dish with water inside to prevent seed dehydration, and the seeds germinated at 20°C in an incubator with a 12:12-h light:dark photoperiod. We recorded the number of germinated seeds each day. If there was no increase in the number of germinated seeds for five consecutive days in the Petri dishes, we would cease observation and record the number of seeds that did not germinate in the Petri dishes to calculate the seed germination rate (number of germinated seeds/total number of seeds) in each of the small Petri dishes.

In May 2012, 1000 seeds from each treatment were sown in five plots, with 200 seeds in each plot, in our experimental field ($36^{\circ}37' \text{N}$, $101^{\circ}45' \text{E}$, 2200 m a.s.l.) in Xining (Qinghai, China). In 2013, only 8–16 plants survived for each treatment, thus we pooled the plants from the five plots for each treatment. We measured the plant height and counted the flower number on each plant, and then harvested them. In the laboratory, all the plants were put in paper bags separately, and then dried at 60°C for 48 h. The dried plants were weighed using an electronic balance (0.1 mg) to get the biomass of each plant.

Inbreeding depression (δ) of *C. pulmonarium* under netting and robbing conditions was estimated at six stages: seed number, seed mass, seed germination, plant height, flower number, and plant biomass. Independent samples *t*-test was used to compare the differences in performance of each life history stage between selfed and outcrossed progenies. The magnitude of ID at each stage was calculated using $\delta = 1 - (W_s/W_o)$, where W_s and W_o were the fitness of selfed and outcrossed progenies, respectively. Cumulative ID was calculated by multiplying fitness values for each cross-type across the life stage and then applying the formula above.

2 Results

2.1 Self-compatibility

No significant difference in seed-set ratio was found between hand selfed and outcrossed flowers in 2001 ($t = 0.32$, $d.f. = 16$, $P = 0.75$) and 2009 ($t = 0.06$, $d.f. = 56$, $P = 0.95$), indicating that *Comastoma pulmonarium* is fully self-compatible.

2.2 Contributions of self-pollen and alien pollen to seed-set ratio

Netted flowers produced as many seeds as control flowers, but the seed-set ratio of emasculated flowers subjected to open pollination was significantly less than that of control and netted flowers (Fig. 1). In 2009, only treatment affected the seed-set ratio significantly ($F_{2,4} = 1554.88$, $P < 0.001$), but population ($F_{2,4} = 2.46$, $P = 0.09$) and treatment \times population interaction ($F_{4,148} = 1.37$, $P = 0.25$) had no significant effect on the seed-set ratio, so we pooled the data in the three populations in 2009. Thus, we further compared the seed-set ratio using a two-way ANOVA with treatment as the fixed factor and year as the random factor. The seed-set ratio of *C. pulmonarium* was affected significantly by treatment ($F_{2,2} = 1683.40$, $P < 0.001$), but not by year ($F_{1,3} = 2.04$, $P = 0.12$) or treatment \times year interaction ($F_{2,243} = 0.40$, $P = 0.67$). These results suggested *C. pulmonarium* produced seeds mainly through autonomous selfing in the study site, and the low seed-set ratio of emasculated flowers subjected to open pollination might result from ineffective pollinators, such as thrips, because we had observed thrips in flowers when we measured the volume of nectar.

2.3 Observations of visitors and nectar production

During our observations of flower visitors, only *Bombus kashmirensis* visited flowers of *C. pulmonarium* for nectar. However, *B. kashmirensis* bit holes in the corolla to get nectar, without entering the corolla tube or touching the anthers/stigma, suggesting that bumblebees were robbers instead of pollinators. Within

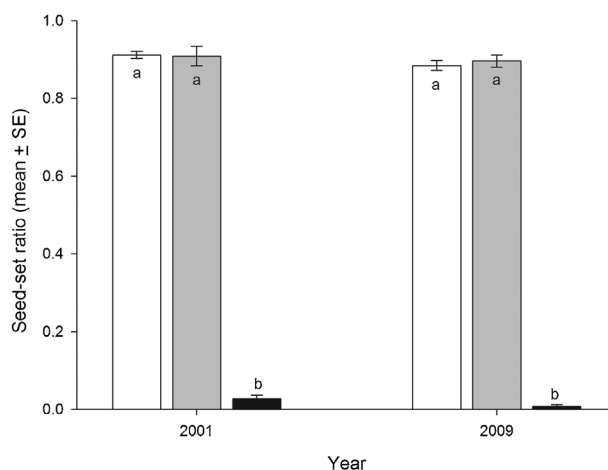


Fig. 1. Contribution of self-pollen and alien pollen on seed-set ratio of *Comastoma pulmonarium* in 2001 and 2009. White, gray, and black bars indicate open pollination, netting without emasculatation, and emasculatation without netting, respectively. Bars with the same letter suggest no significant difference in each year.

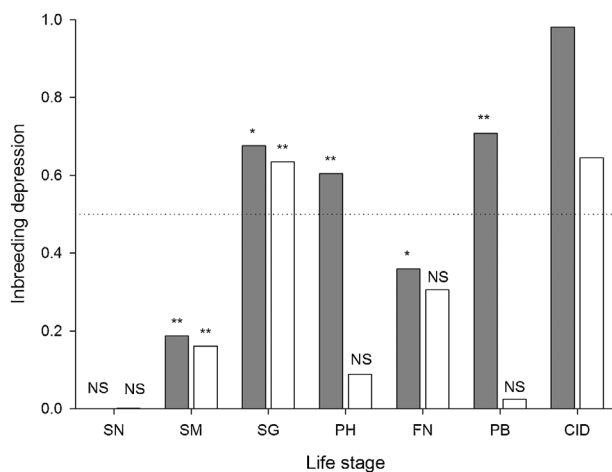


Fig. 2. Inbreeding depression of *Comastoma pulmonarium* from netted (filled bars) and robbed (open bars) conditions. Comparisons were carried out between selfing and outcrossing treatments. Dotted line indicates the theoretically predicted threshold below which selfing should evolve. * $P < 0.05$; ** $P < 0.01$. CID, cumulative inbreeding depression; FN, flower number; NS, no significant difference; PB, plant biomass; PH, plant height; SG, seed germination; SM, seed mass; SN, seed number.

1 day, the nectar volumes in the three populations were $5.84 \pm 0.27 \mu\text{L}$ (mean \pm SE) with sample size $n = 60$, $3.84 \pm 0.27 \mu\text{L}$ ($n = 40$), and $4.6 \pm 0.38 \mu\text{L}$ ($n = 40$). Therefore, the nectar of *C. pulmonarium* was consumed exclusively by robbers.

2.4 Inbreeding depression

Under both netting and robbing conditions, the difference between selfed and outcrossed progenies was significant in the stages of seed mass and germination percentage, but not in the stages of seed number, plant height, flower number, or plant biomass, suggesting that ID occurred in the stages of seed mass and germination (Fig. 2). Overall, in comparison with robbing conditions, the magnitude of ID in each life history stage, except seed number, and the cumulative ID were higher under netting conditions (Fig. 2). The cumulative ID was 0.98 and 0.64 in netting and robbing conditions, respectively, both of which were much higher than 0.5 (Fig. 2).

3 Discussion

Our study suggests that autonomous selfing provides reproductive assurance for *Comastoma pulmonarium* with nectar secretion but no pollinator visitation in the studied populations. However, the ID in *C. pulmonarium* exceeds 0.5, the predicted threshold below which selfing would evolve (Lande & Schemske, 1985; Kalisz et al., 2004; Cheptou &

Donohue, 2011), and thus outcrossing should evolve under strong ID in this species. Below we discuss the evolution of selfing and ID in the complete absence of pollinators in *C. pulmonarium*.

3.1 Evolution of selfing in *C. pulmonarium*

Providing reproductive assurance is one of the most widely accepted explanations for the evolution of selfing in plant species, especially for those inhabiting environments with unpredictable pollinator services (Kalisz et al., 2004; Zhang & Li, 2008; Duan et al., 2010). Nevertheless, this explanation has been challenged by several studies (Eckert & Schaefer, 1998; Herlihy & Eckert, 2002; Vaughton & Ramsey, 2010). However, it should be noted that these results were based on studying plants with pollinator visitations, thus pollen and/or ovule discounting occurred in these species. In *C. pulmonarium*, no pollinator was observed to visit the flowers in 2001 or 2009, therefore, no pollen or ovule discounting occurred in this species under complete absence of pollinators (Lloyd, 1992), and ID should be the only factor preventing the evolution of selfing in this alpine species.

In *C. pulmonarium*, the cumulative ID exceeds 0.5, which is strong enough to prevent the evolution of selfing, thus outcrossing should evolve. However, in the studied populations, results from both the mating system and observations suggested that no pollinator visited *C. pulmonarium*, thus the nectar was exclusively consumed by robbers. Accordingly, the complete pollinator failure might have facilitated the evolution of selfing in *C. pulmonarium*, even with strong ID, suggesting a “better than nothing” role of autonomous selfing to provide reproductive assurance in the absence of pollinators (Duan et al., 2010) and high ID (Ruan et al., 2009).

3.2 Evolution of ID in *C. pulmonarium*

As we have mentioned above, no pollen or ovule discounting occurred in *C. pulmonarium*, in that no pollinator visited flowers of this species in the studied populations; thus, ID could have played an important role in the evolution of autonomous selfing in this alpine annual. In highly inbred populations, after continuous selfing, deleterious recessive mutation should be effectively purged as the deleterious genotypes become exposed to natural selection (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987). In *C. pulmonarium*, our results of predominant selfing in 2001 and 2009 suggested that this species might have produced seeds by autonomous selfing at the study site. However, the cumulative ID exceeds 0.5 in both netting and robbing conditions, which indicated that outcrossing

might be expected to be involved in the populations under a good pollination environment (Cheptou & Donohue, 2011), but this speculation needed to be argued in future studies. Importantly, in the late life history, the magnitude of ID was reduced significantly to below 0.5, suggesting nectar robbery weakened the magnitude of ID to a large degree. However, due to the strong ID in the early life history in *C. pulmonarium* (Zhang et al., 2011), the cumulative ID was above 0.5, and thus the decrease in the magnitude of ID was not strong enough to facilitate the evolution of selfing.

4 Conclusions

This study concentrated on the evolution of autonomous selfing in an alpine annual on the Qinghai–Tibet Plateau, providing one of the few cases of a selfed annual with strong ID. Our results suggested that autonomous selfing could provide reproductive assurance for species in the complete absence of pollinators, even with a high magnitude of ID, supporting the “better than nothing” role of the evolution of autonomous selfing.

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