

# Delayed Selfing in an Alpine Biennial *Gentianopsis paludosa* (Gentianaceae) in the Qinghai-Tibetan Plateau

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## Abstract

Delayed selfing could provide ovules with an opportunity to be fertilized as a means of “pollination assurance” before the flowers wilt. It could, thus, be regarded as an adaptation to unpredictable pollinator environments. Within the alpine biennial *Gentianopsis paludosa*, the showy flowers and herkogamy at the early stage of a flower’s life cycle may favor outcrossing. As the flower ages, anthers contact the central stigma due to the elongation of all filaments, resulting in autonomous selfing. Flower visitors are extremely rare in a high altitude population; and examination of the mating system indicates that *G. paludosa* is self-pollinated under natural conditions in this population. While at the lower altitude, the bumblebee visitation rate is relatively high but possibly unreliable. Stigma receptivity is the highest on the third day of anthesis, and decreases thereafter. Pollen viability is the highest when flowers open, and gradually decreases later. Self-pollination of *G. paludosa* occurs at the late stage of a flower’s lifecycle when stigma receptivity and pollen viability have both decreased, suggesting delayed selfing and assurance of seed production. This delayed selfing could assure seed production under the constraints of pollinator scarcity, but ensure outcrossing when pollinators were available. Such a flexible pollination mechanism is highly adaptive in the alpine environment of the Qinghai-Tibetan Plateau.

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## Introduction

The shift from outcross to self-pollination is one of the most common evolutionary trends in flowering plants (Stebbins 1970; Wyatt 1988; Barrett 1998). The constraint to set seeds when pollinators and/or potential mates are scarce is proposed as being the principal selection pressure for the evolution of self-pollination (Baker 1955; Lloyd and Schoen 1992; Fausto et al. 2001; Gómez 2002; Wang et al. 2005; Zhang and Li 2008; Chen et al. 2009). Nevertheless, both male and female fitness

may be reduced because of inbreeding depression and pollen and/or ovules discounting when pollen and/or ovules are selfed (Uyenoyama et al. 1993; Barrett 1998; Harder and Routley 2006). Delayed selfing could prevent these limitations and provide reproductive assurance due to the provision of the opportunity for outcrossing before self-pollination occurs, as well as the assurance of ovule fertilization at the end of anthesis (Lloyd 1992; Barrett and Harder 1996; Kalisz et al. 1999; Brunet 2005). This selfing mechanism, therefore, is selectively advantageous where pollinators and/or pollen are limited, and

it is expected to benefit both male and female functions when the absence of pollinators reduces both seed production and pollen-mediated mating opportunities (Holsinger 1991; Lloyd 1992; Kalisz and Vogler 2003; Kalisz et al. 2004).

The most common delayed selfing is “corolla dragging” whereby a withered corolla drag anthers across the stigma (e.g., Dole 1990; Sun et al. 2005; Qu et al. 2007). Another delayed selfing mechanism was recently discovered in a tree-living orchid (*Holcoglossum amesianum*), in which the flower turns its anther through 360° to insert pollen into its own stigma cavity without the aid of a pollinating agent or medium (Liu et al. 2006). In *Collinsia verna*, the anthers mature at different times and early anthers maintain spatial separation from the stigma, thus preventing self-pollination; however, contact of the late-developed anthers with the stigma results in delayed selfing (Kalisz et al. 1999). Although delayed selfing is considered to be common in angiosperm species (see review by Fenster and Martén-Rodríguez 2007), this mechanism has rarely been reported in alpine plants. In alpine environments, levels of insect diversity, abundance and activity are low, which ultimately leads to limited pollination of alpine plants and promotes a prevalence of the selfing system (Körner 1999). Nevertheless, the benefits of reproductive assurance through self-pollination might be outweighed by the severe inbreeding depression and pollen and/or ovule discounting (Herlihy and Eckert 2002; Brunet 2005), especially in the arid alpine environments where the magnitude of inbreeding depression is more rigorous than in the low-altitude region (Dudash 1990). In the alpine ecosystems, therefore, the delayed selfing mechanism would be expected to be highly adaptive.

The present study aimed to examine whether delayed selfing occurs in *G. paludosa* Ma (Gentianaceae), an alpine biennial in the Qinghai-Tibetan Plateau, which may have served as an effective mechanism of the reproductive assurance under this harsh pollination environment. Although the showy flowers of this species appear to be adapted to animal pollination, insect visits were rarely observed under the natural conditions during our preliminary observations of the populations in the high altitude before the present formal study. However, fruit sets were high, which might be the result of self-pollination. In this study, therefore, we address the following questions: (i) Does self-pollination occur in *G. paludosa*? (ii) If so, what is the mechanism that promotes it? (iii) Could the mechanism of self-pollination provide reproductive assurance for this species?

## Results

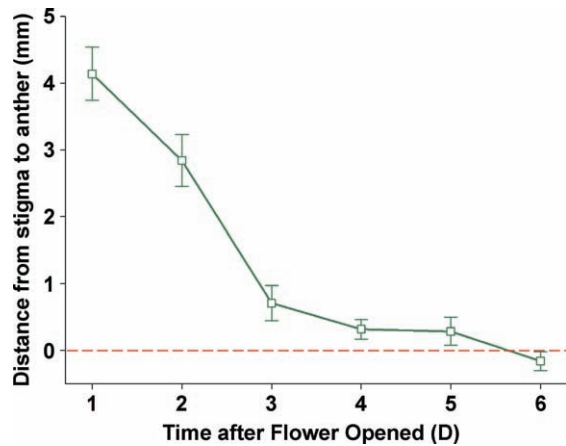
### Floral characteristics and variable herkogamy during flower development

*G. paludosa* begins to flower in early July. Flowers of this plant are blue-purple and have a showy appearance (Figure 1). Its stigma is bifurcate before flower-opening (Figure 1A), and four anthers with the same height begin to shed pollen when flowers open (Figure 1B). At this stage, the stamens are shorter than the pistil (Figures 1B, 2). About 5 days after anthesis commencement, the height of the stamens equals or exceeds the height of the pistil due to the elongation of all filaments (Figure 2), resulting in stigma-anther contact (Figures 1C, 2). Thus, flowers of *G. paludosa* exhibit herkogamy



**Figure 1.** Flower development of *Gentianopsis paludosa*.

- (A) Flower at bud stage with anthers below the stigma. Anthers are not yet mature.  
 (B) Mature anthers are positioned below the receptive stigma. Pollen is released at this stage.  
 (C) Filaments have elongated and anthers are now above the position of the stigma. Self-pollen can be deposited on the stigma, but this pollen has reduced viability when compared with pollen from anthers as in (B).



**Figure 2.** Changes in distance from stigma to anthers of *Gentianopsis paludosa* with flower aging.

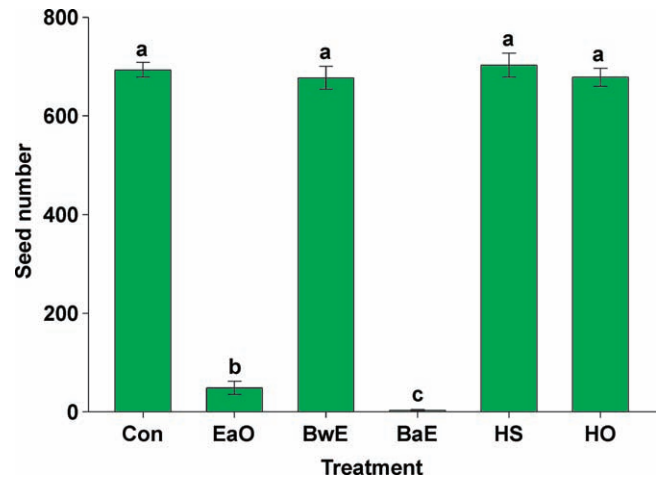
The distance was calculated by subtracting the height of the stamens from the height of the pistil ( $n = 20$ ). Data are presented as mean  $\pm$  1 SE, and  $n$  refers to sample size.

only early in the flower's life cycle, and the distance between the anthers and the stigma decreases gradually during the flower's development (Figure 2), enabling the possibility of self-pollination.

### Mating system

Because of the occasional herbivore in the permanent sample site, some labeled flowers can not be found and thus the sample size is reduced for each treatment. In total, there was no significant difference (all  $P > 0.05$ ) in either fruit set or seed number per fruit for each treatment over the 3 years; therefore, the seed number of each treatment over 3 years was pooled. Fruit set of the bagged and emasculated flowers was as low as  $12.3\% \pm 0.08\%$  ( $n = 81$ ) and fruit from this treatment produced on average only  $3.0 \pm 1.7$  seeds per fruit (Figure 3), suggesting that apomixis does not occur in *G. paludosa*. Of the emasculated and open pollinated flowers ( $n = 84$ ),  $34.5\% \pm 0.07\%$  set fruits and produced  $48.8 \pm 13.4$  seeds/capsule.

The bagged flowers without emasculations ( $n = 83$ ), hand selfing ( $n = 77$ ) and hand outcrossing ( $n = 70$ ), as well as the open pollinated flowers (the control flowers) ( $n = 89$ ), produced high fruit sets (100%) and seed number (ranging between 600 and 800), and no significant differences were detected between any two treatments of control, bagging without emasculations, hand selfing and hand outcrossing (Figure 3). In addition, the fruit sets (100%) and seed number ( $n = 65$ ) are also high (600–800 seeds/fruit) of open pollinated flowers in the Qilian population in the low altitude.



**Figure 3.** Seed number from different treatments in *Gentianopsis paludosa* over three consecutive years.

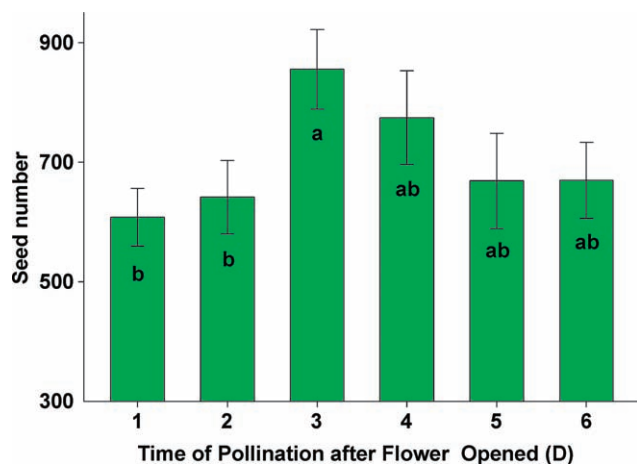
Values with the same letters indicate that the difference is not significant at the 0.05 level. BaE, Bagging after emasculations ( $n = 81$ ); BwE, bagging without emasculations ( $n = 83$ ); Con, control (open pollination) ( $n = 89$ ); EaO, emasculations and open pollination ( $n = 84$ ); HO, hand outcrossing ( $n = 70$ ); HS, hand selfing ( $n = 77$ ). Data are presented as mean  $\pm$  1 SE, and  $n$  refers to sample size.

### Observations on visitors

According to the 2 years of observations (45 h in total) in the high altitude (Haibei) population, only five butterflies visited *G. paludosa*. They visited flowers for short stays (for about 2–3 s) on the corolla lobe; they did not enter the corolla tube. Therefore, butterflies could not be considered as frequent visitors or pollinators of this species. No other insect visitor was observed over the two seasons monitored in the population. However, in the Qilian population, two bumblebees were observed to visit flowers of *G. paludosa* during our observations of the first day (6 h), and no insect was observed in the second day's observation (6 h). The two bumblebees visited 12 flowers in total and the average visiting frequency was 0.025 visits/flower per h. Bumblebees entered corolla tubes, stayed for more than 5 s and their bodies contacted both anthers and the stigma in each. These observations suggested that bumblebees were effective pollinators of *G. paludosa* in this low altitude population, although their visiting frequency was very low. Additionally, we found no secretion of nectar in the bagged flowers in the two populations, suggesting that the four nectaries of *G. paludosa* had degenerated without producing nectar.

### Stigma receptivity and pollen viability

Seed number of manually pollinated flowers with pollen from newly opened flowers, was low for the first and second day,



**Figure 4.** Seed number from hand outcrossed flowers during the different floral duration (days) of *Gentianopsis paludosa*, indicating the changes of the stigma receptivity when flower ages.

Sample size for each day is 15, and values with the same letters mean that the difference is not significant at the 0.05 level. Data are presented as mean  $\pm$  1 SE, and  $n$  refers to sample size.

peaked on the third day, and then decreased in the following days (Figure 4), suggesting variations in stigma receptivity with flower aging and decreased stigma receptivity when anther-stigma contacted in *G. paludosa*. Moreover, there was no significant difference in seed number per capsule between selfing and outcrossing flowers of the same age (T-test,  $P > 0.5$ ).

We observed significant variations in the pollen germination rate, an indicator of pollen viability, in the different days ( $F = 9.56$ ,  $P < 0.01$ ). Pollen germination rate was highest on the first day and decreased gradually (Figure 5), indicating decreased pollen viability with flower aging.

## Discussion

*G. paludosa* exhibits herkogamy at the early stage of flower opening, and the spatial separation between anthers and stigma is sufficient to prevent self-pollination, although the anthers are mature and pollen is shedding at this stage. The opportunity for cross pollination in the early stages of flower development is further supported by incomplete protandry in *G. paludosa* (Liu and Ho 1997). As indicated in the present study, the stigma receptivity reached the highest on the third day after flower opening, obviously later than the peak of pollen viability (newly-opened flowers), suggesting that not all ovules are mature when all pollens are viable at the early flowering stage.

Bumblebees are the dominant pollinators of alpine plants (Bingham and Orthner 1998), and the bumblebees could be



**Figure 5.** Pollen germination rate from flowers during the different floral duration (days) of *Gentianopsis paludosa*, indicating the changes of the pollen viability when flower ages.

Sample size for each day is 8, and values with the same letters mean that the difference is not significant at the 0.05 level. Data are presented as mean  $\pm$  1 SE, and  $n$  refers to sample size.

an effective pollinator of this species if they were available because we found that bumblebees visited *G. paludosa* in the low altitude population with a low frequency. However, we failed to observe bumblebee visits to this species in the two years in the high altitude population although they did pollinate the other gentians in this region (Duan and Liu 2003, 2007; Duan et al. 2005, 2007a; Zhang et al 2006). The bumblebees visited these gentians mainly for nectar rewards (Kozuharova 1999). The non-functional nectaries of *G. paludosa* may discourage their visits in the high altitude because bumblebees need more energy and visit more frequently on the species with abundant nectars (Bingham and Orthner 1998). The infrequent bumblebee visitation in the low altitude may lead to outcross pollen limitation of *G. paludosa* as observed here, which may drive the evolution of self-pollination.

Our experiments on mating systems suggested that *G. paludosa* is self-compatible (Figure 3). In addition, the bagged flowers without emasculation produced a number of seeds via autonomous self-pollination, similar to those of the control flowers (Figure 3). However, the fruit set ( $34.5\% \pm 0.07\%$ ) and seed number ( $48.8 \pm 13.4$ ) of the emasculated and open pollinated flowers were extremely low, suggesting little pollination contributed by the effective pollinators (for example, bumblebees). The low fruit/seed sets in this experiment may derive from the rare pollinations by ineffective pollinators (e.g. thrips and ants). It is obvious that these small visitors play an extremely minor role in seed production of the whole population because both fruit success and seed number of the emasculated and open pollinated flowers were far lower than those

of the controlled flowers (Figure 3). These mating experiments together suggest that the high natural fruit set and seed number of this species result mainly from the self-pollination because flower visitors were nearly unavailable in the high altitude population or infrequent in the low altitude population. We did not examine seed discounting and inbreeding depression of the self-pollination in *G. paludosa*; however, the lack or shortage of the frequent pollinators indicates that self-pollination must have provided a reproductive advantage for this species, even if inbreeding depression does occur (Lloyd 1992).

Our observations of the floral developments on *G. paludosa* suggested that floral herkogamy prevented selfing and encouraged outcrossing at the early flowering stage. However, with flower aging (the fifth and sixth day), anthers contacted the central stigma and self-pollination occurred when both pollen viability and stigma receptivity have greatly been decreased (Figures 4, 5). It is obvious that this self-pollination occurs after cross-pollination, which is typical of “delayed selfing” (Dole 1990; Ippolito and Armstrong 1993; Sun et al. 2005; Qu et al. 2007; Duan et al. 2007b). The benefits of reproductive assurance from self-pollination are usually considered to be outweighed by severe seed discounting and inbreeding depression (Herlihy and Eckert 2002) although the inbreeding depression may be weakened after continuous selfing (Takebayashi and Delph 2000). However, as repeatedly pointed out previously (Kalisz et al. 1999, 2004; Kalisz and Vogler 2003), the delayed selfing incurs less cost because this mechanism ensures outcrossing first if pollinators are available and assure the seed production via selfing later when pollinators are unavailable. Undoubtedly, this flexible pollination mechanism developed for the biennial *G. paludosa* and the other species of the same genus (Duan et al. 2007b) should be highly advantageous in the alpine environments of the Qinghai-Tibetan Plateau where effective pollinators (for instance, bumblebees) are always scarce or unstable (Duan et al. 2007a).

## Materials and Methods

### Study site

Most of our work was carried out between early July and early August from 2001 to 2003 in the population at the Haibei Alpine Meadow Ecosystem Research Station of the Chinese Academy of Sciences, which is located in the northeast Qinghai-Tibetan Plateau (lat. 37°29′–37°45′ N, 101°12′–101°23′ E, 3 200 m a.s.l.) (hereafter the Haibei population). This population is in the permanent sample site of the Haibei station, and the alpine meadow under investigation is dominated by *Kobresia humilis*, while *Gentianopsis paludosa* occurs as a subdominant herb.

Observations on flower visitors were also carried out in another population around Qilian county at a lower altitude of

2 600 m a.s.l. in 2003 (hereafter the Qilian population), about 50 km from the Haibei population. In September, 2009, we revisited this population and collected some fruit randomly to examine the seed production in naturally pollinated flowers.

### Studied species

*G. paludosa* is a biennial herb inhabiting alpine environments in the ranges from 1 180 m to 4 900 m a.s.l. The plant ranges from 3.5 cm to 40 cm in height and blooms from early July until early August; and it carries  $3.0 \pm 2.1$  ( $n = 135$ ) single terminal flowers on different stalks. The flower is about 4–5 cm in length and has a long corolla tube and four horizontally spreading lobes (Figure 1), and four projecting nectarines, which are suborbicular and nodding, and positioned opposite the lobes at the base of the corolla tube (Ho and Pringle 1995).

### Flower development

To investigate the changes in flower characteristics during flower opening, 20 flower buds, each from different plants, were labeled in the Haibei population in 2002. At the commencement of flower opening, we removed one lobe of the corolla and, every day, we measured the heights of the stamens and pistil using a hand-held digital calliper until the anthers of all of the selected flowers touched the stigma. The heights of the stamens and the pistil were measured from the bottom of the ovary, and the distance from the stigma to the anthers was calculated by subtracting the height of the stamens from the height of the pistil to eliminate error in measuring. By comparing the time interval from flower opening to anther-stigma touch in untreated flowers as a control, we ensured that the removal of the flower lobe did not affect the process of flower development.

### Mating system

In the Haibei population, we selected 150 buds at random from different individuals that were subjected to the following treatments (30 flowers per treatment): (1) emasculation and open pollination; (2) bagging without emasculation; (3) bagging after emasculation; (4) hand selfing and (5) hand outcrossing. For 1–3, all of the treatments were carried out before the flower opened. In treatments 4 and 5, flowers were emasculated and bagged at the bud stage before the dehiscence of the anthers. After the flowers had been open for 5 days, they were hand-pollinated with recently dehisced anthers (peak of pollen viability) from the same plant in Treatment 4 and in Treatment 5 from another plant 10 m away from the receptive plant; the stigma was brushed with anthers and loaded with a heavy pollen load, presumably enough to fertilize all of the ovules. After artificial pollination, the flowers were bagged again. Another

30 buds were selected and left for open pollination (control). To prevent the effect of position on the ovule number in one plant, only the terminal flowers were selected for this mating system experiment. All of the labeled flowers were collected before fruit dehiscence and taken back to the laboratory to count the number of mature seeds per fruit, because the unfertilized ovules and aborted seeds were almost invisible. These experiments were replicated over 3 years in order to test the year-to-year variation of the mating system in this high altitude population. Additionally, we also checked fruit and seed sets of the Qilian population.

### Observations on visitors

The floral visitors of *G. paludosa* were recorded in the Haibei population from 26–31 July 2002; and 23–26 July 2003. Each day before observations, 20 neighboring open flowers on different individuals were selected at random and labeled. One researcher observed these flowers from 08.30 to 12.00 hours in 2002 and from 09.00 to 15.00 hours in 2003. In total, we carried out observations for 45 h in the 2 years. In the Qilian population, visitors were observed in 40 neighboring flowers in one patch over 2 d between 08.30 to 15.00 hours, 12 h in total. During these observations, we stayed 2 m away from the focal flowers to observe all of the floral visitors without disturbing their foraging behavior. The weather was warm and air temperatures were relatively high (above 20°C). Because corolla lobes of *G. paludosa* usually close after 15.00 hours in the afternoon due to the drop in temperature, we did not carry out observations after that time. We recorded visitor species, behavior in the flower and visiting times of each species. In addition, we also bagged 20 flowers at 08.00 hours in 2003 in the two populations, and on the next day at 08.00 hours, and checked the nectar volume using microcapillaries.

### Stigma receptivity

Stigma receptivity was assessed by pollinating 15 emasculated flowers of different ages with pollen of newly dehisced anthers from other plants. Flowers were bagged before and after the treatment, and the averaged seed number was used to assess stigma receptivity in relation to the flower age. Seed production was used as a criterion for stigma receptivity of the tested flowers. In order to assess the effect of self versus outcross pollen on stigma receptivity, another group of flowers was hand self-pollinated with pollen from the same individual on the first, third, and fifth days (15 flowers for each day in this group).

### Pollen viability

Pollen viability was assessed by the pollen germination rate in a sucrose solution with a concentration of 10% by weight (Dafni

et al. 2005) (after a preliminary experiment showing high pollen germination under this concentration). Eight bagged flowers of different ages (eight flowers for each stage) were used to examine pollen germination. To prevent the pollen loss due to self-pollination in the late stage of flower opening, we removed the stigmas of all bagged flowers. All of the anthers of one flower were collected and put in a sucrose solution at 25°C. The pollen germination was recorded after 24 h. For each flower, pollen grains of all anthers were fully suspended in 1.5 mL of sucrose solution before counting. We used a pipette to get 10 µL of solution to count the number of germinated and non-germinated pollen grains on the slide under a microscope, and each flower was replicated 10 times.

### Statistical analysis

One-way ANOVA and post hoc-least significant different (LSD) were used to compare: (i) seed number among different treatments over the 3 years; and (ii) seed number from hand outcrossed flowers and pollen germination rate at different flower ages. Prior to comparison, all of the data were tested for normality. Data analyses were performed and were generated using SPSS software version 11.0 (SPSS, Chicago, IL, USA). Data are presented as mean  $\pm$  1 SE, and *n* refers to sample size.

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