

Pollination ecology and its implication for conservation of an endangered perennial herb native to the East-Himalaya, *Megacodon stylophorus* (Gentianaceae)

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Background and aims – *Megacodon stylophorus* (C.B. Clarke) Harry Sm. is an endangered herb native to the East-Himalaya and the Hengduan Mountains, south-west China. We studied reproductive ecology of this species to explore the factors that are critical for its survival and conservation.

Methods – We conducted breeding system experiments during 2008 and 2009 in two populations located in the Hengduan Mountains.

Key results – The flowers are protandrous and herkogamous, and thus insects are necessary for pollination and seed production. Bumblebees were observed to be the most frequent visitors, and their visitation rates were lower at the higher altitude. Seed production in both populations fluctuated annually, possibly due to pollen limitation, which occurred in both populations in the two years.

Conclusions – The pollination level is only marginally responsible for the rarity of this alpine species. We suggest that *in situ* conservation measures should be given the highest priority at present, considering the strong genetic differentiation among populations.

Key words – *Megacodon stylophorus*, breeding system, bumblebee pollination, the Hengduan Mountains, *in situ* conservation.

INTRODUCTION

The Hengduan Mountains along with the southeastern part of the Qinghai-Tibetan Plateau (QTP) and the adjacent areas comprise one of only two biodiversity ‘hotspots’ in the northern hemisphere (Myers et al. 2000). In this region, organisms seem to have diversified extensively, resulting in an extraordinary abundance of endemic species (e.g. in Wang et al. 2005, Liu et al. 2006, Zhang et al. 2009). However, the high potential instability and inherent vulnerability of mountain ecosystems render the Himalayan region an ecologically fragile biogeographic zone (Rodgers & Panwar 1988). Loss of habitat by deforestation and the pressure of excessive grazing in high altitude pastures now threaten the survival of endemic and rare plants in the Hengduan Mts. (Kala 2000, Rai et al. 2000); many endemic species in this hotspot are becoming endangered due to increasing human activities and consequent habitat deterioration in this region (e.g. in Ge et al. 2005, Zheng et al. 2008). Hence there is an urgency to undertake conservation initiatives of the endemic plants in this region.

Understanding the reproductive ecology of endangered species will promote effective conservation efforts. Hence studies on the pollination ecology of plants could contribute to evaluating their conservation status and finding appropriate ways of conserving the species (Carlsen et al. 2002, Evans et al. 2004), especially for plants which need pollinators for sexual reproduction, since their pollinators could be affected greatly by human activities. Furthermore, biodiversity hotspots are experiencing greater declines in pollinator abundance and diversity than those of less species-rich areas. Therefore, pollen limitation is also more severe in self-incompatible plants inhabiting biodiversity hotspots than other regions (Vamosi et al. 2006), which can lead to an increasing extinction risk of plant species depending on pollinators for seed production, potentially threatening the maintenance of biodiversity. Accordingly, understanding the pollinator characteristics of these endangered species in biodiversity hotspots is necessary for initiating effective conservation.

Megacodon is a small genus (including only two species, *M. stylophorus* (C.B. Clarke) Harry Sm. and *M. veno-*

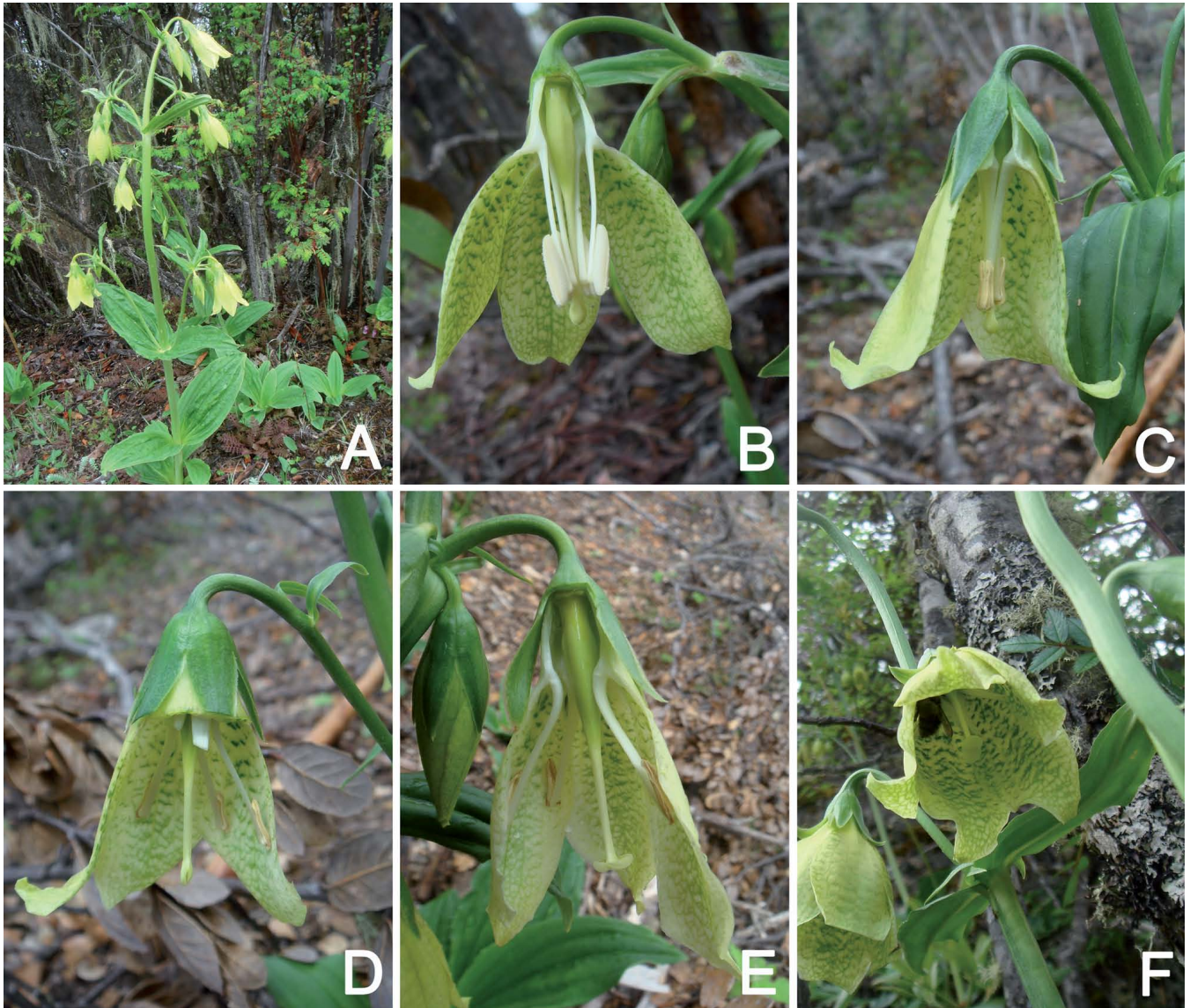


Figure 1 – Flower characteristics of *M. stylophorus*: A, habit of *M. stylophorus* with nodding buds and flowers; B, anthers are shedding pollen grains; C, anthers have terminated shedding pollen grains; D, anthers without pollen grains have moved outwards against corolla, but the two stigma lobes are still closed; E, the two stigma lobes are open, with anthers against corolla; F, bumblebee visiting a flower of *M. stylophorus*.

stylophorus (Hemsl.) Harry Sm.) of family Gentianaceae, subtribe Swertiinae (Gentianaceae-Gentianeae) (Yuan & Küpfer 1995, Chassot et al. 2001, von Hagen & Kadereit 2002, Xue & Li 2005). *M. stylophorus* is native to the East-Himalaya and mainly distributed in the Hengduan Mountains. This species is locally abundant, but its populations are scattered as isolated patches across mountains throughout this region. We found that habitats of this species were destroyed by logging by local people during our field investigations, and thus *in situ* conservation should be carried out, but before which pollination ecology should be performed to acquire the preliminary reproductive traits of this species. Hence, pollination ecology of *M. stylophorus* was studied in two natural populations at different altitudes addressing the following questions: (1) Is selfing possible in *M. stylophorus*? (2) What are the potential pollinators of this species? (3) Is its seed

production pollen-limited? And if so, does the magnitude of pollen limitation differ between high and low altitude populations? (4) What are the implications of the pollination characteristics of *M. stylophorus* for its conservation?

MATERIAL AND METHODS

Study species and sites

Megacodon stylophorus is a perennial herb native to the East-Himalaya, usually growing in montane habitats beside streams, shrubs or forest margins at altitudes ranging from 3000 m to 4400 m. The height of this plant is 30 to 60 cm, sometimes it can reach 100 cm. The inflorescence is a thyrses with 3 to 9 large, pendant flowers (fig. 1A). The corolla is campanulate in pale yellow-green, with brown veins. With-

in a flower, there are five anthers and one stigma, and the nectaries are placed in a whorl around the base of the gyno-phore. The capsules are ovoid-ellipsoid, 3 to 4 cm long and 2.2 to 2.5 cm in diameter, with oblong and rugose seeds. *M. stylophorus* flowers in June and July, and sets fruit from July to September (Ge et al. 2005, Ho & Pringle 1995).

Experiments were carried out from early June to late August in 2008 and 2009, in southwest Tibet. The two populations were located around the Lulang township (3200 m a.s.l., the low altitude population) and Linzhi Alpine Forest Ecosystem Research Station (3900 m a.s.l., the high altitude population), respectively. More than 200 individuals were found in each population.

Methods

Flower development – In the low altitude population, we randomly selected fifteen plants of *M. stylophorus* spread over the whole population. Flower development of all the buds was observed every two hours from 7:00 to 21:00 until all the flowers wilted. According to our preliminary observations, flowers of *M. stylophorus* showed dichogamy and herkogamy, and thus we recorded the time of flower opening, pollen shedding, termination of pollen shedding, movement of stamens, stigma lobe opening and corolla wilting. Using these data, we calculated the duration of the male phase, which was defined from the time of pollen shedding to termination of pollen shedding, and the female phase, which was defined from the initial opening of the stigma lobes to the stigma turning brown.

Pollen and ovule number – Ten buds from random plants were collected in 2008 and fixed in a solution of formalin, acetic acid and alcohol (FAA). Number of ovules was counted under a stereomicroscope. To assess the number of pollen grains, the five anthers per bud were squashed and diluted in 70% ethanol with a drop of detergent to obtain 5 ml suspension (Dafni 1992). The suspension was thoroughly shaken, and 10 replicates of 10 μ l were sampled with a micropipette. The number of pollen grains was counted on the slide glass under a microscope in order to determine the total number of pollen grains for each floral bud. The pollen/ovule ratio was also calculated.

Self-compatibility – To examine self-compatibility of this species, thirty pairs of opposite flower buds were selected and labeled. In this and the following experiments, we selected paired flowers to avoid position effects on the ovule number and resource availability, and each pair of flowers was on a different plant. We emasculated the labeled flowers during the period between flower opening and stigma dehiscence, and then isolated them with fine nylon nets (1-mm mesh) that effectively exclude small insects. When the stigma was open, hand pollination on each pair of the opposite flowers was conducted, one with pollen grains from the same plant, and the other with pollen grains from a different plant which was 20 meters away from the receptor plant. All the flowers were netted again after hand pollination, and fruits were collected before capsule dehiscence to determine the number of mature seeds per fruit, and a paired-sample *t*-test was used to compare the seed numbers of paired flowers.

Capacity for autonomous selfing and apomixis – We randomly labeled 60 flower buds and divided them into two groups in 2008 and 2009. For one group ($N = 30$), flower buds were netted with nylon nets to examine whether *M. stylophorus* could produce seeds via autonomous selfing. For the other group ($N = 30$), flowers were emasculated before stigma opening and netted to determine whether apomixis could happen. All the fruits were collected before dehiscence and carried back to the laboratory to count the number of mature seeds per fruit.

Additionally, thirty pairs of opposite flower buds were selected and labeled. For each pair of flowers, one was emasculated after flower opening but before stigma opening, and the other was left to be pollinated naturally as a control. Also, the fruits were collected before dehiscence for counting the number of mature seeds per fruit, and a paired-sample *t*-test was used to compare the seed numbers of the paired flowers.

Pollen limitation – To examine whether seed production of *M. stylophorus* is pollen limited, thirty pairs of opposite flowers were selected. When the stigma was open, one flower of each pair was artificially pollinated with outcrossing pollen grains from multiple donors which were at least 10 meters away from the receptor plant, and the other flower was treated as a control and only pollinated naturally. Ripe fruits were collected before dehiscence and carried back to the laboratory to determine seed number, and a paired-sample *t*-test was used to examine the difference in seed number between the treated and control flowers after the seed number was log-transformed. The magnitude of pollen limitation was calculated by $(1 - N_c / N_s)$, where N_c and N_s are seed numbers of control flowers and hand pollinated flowers, respectively. A two-way ANOVA including population and year as fixed factors was used to compare the difference in the magnitude of pollen limitation.

Pollinator visitation rate – Potential pollinators from the flower visitors of *M. stylophorus* were determined. On each of ten continuous sunny days without strong wind, we selected fifteen flowers and recorded the flower phase (male or female), and then observed these flowers from 8:00 to 18:00, during which all the flower visitors were recorded as well as their activities on the flower. We would stop observing the flower visitors if rain occurred. Any visitor which touched the anthers and/or stigmas of *M. stylophorus* during its visit was considered as potential pollinator. Visitation rates of the potential pollinators were also calculated (visits/flower/hour). All the potential visitors were captured and brought back to experts at Kunming Institute of Zoology, Chinese Academy of Sciences, for identification. A three-way ANOVA including flower phase (male or female), population and year as fixed factors was used to test the difference in pollinator visitation rate.

RESULTS

Flowering phenology

Flowers of *M. stylophorus* were nodding in flowering and bud stage (fig. 1A), and became erect in the fruiting stage. In total, the flowering processes of 56 flowers were observed on fifteen different plants. The male phase of the flower (pol-

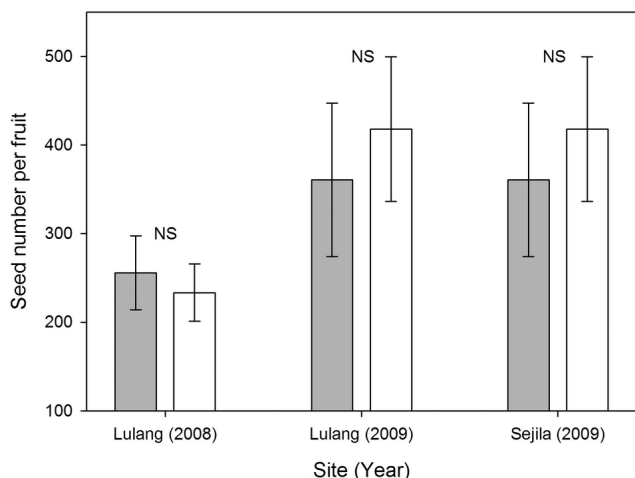


Figure 2 – Seed number of opposite flowers subjected to hand selfing (filled bars) and hand outcrossing (open bars), respectively. Data is shown with mean \pm 1 SE. NS indicates no significant difference.

len started shedding after 1.3 ± 0.1 days of flower opening and stigma remained closed, fig. 1B) lasted 5.3 ± 0.2 days. When pollen shedding was completed, stigma lobes were still found to be closed (fig. 1C). After 0.9 ± 0.1 days, anthers with the filaments moved outwards to the corolla and finally leaned against it (fig. 1D). When the two stigma lobes were open (fig. 1E), the female phase started and the color of the stigma turning green to brown indicated the end of stigma receptivity. The female phase lasted 7.6 ± 0.3 days.

Pollen and ovule number

In the low altitude population, the number of pollen grains ranged from 4.08×10^5 to 6.81×10^5 ($5.52 \times 10^5 \pm 0.38 \times 10^5$, mean \pm SE), and the numbers of ovules ranged from 300 to 499 (369 ± 33). In the high altitude population, the number of pollen grains ranged from 5.19×10^5 to 7.80×10^5 ($6.70 \times 10^5 \pm 0.61 \times 10^5$), and the number of ovules ranged from 166 to 524 (380 ± 72). Therefore, the pollen/ovule ratio was 1569 ± 170 and 1974 ± 306 in the low and high altitude populations, respectively. No significant difference was found between the two populations in pollen number ($t = 1.63$, $df = 18$, $P = 0.13$), ovule number ($t = 0.17$, $df = 18$, $P = 0.88$) and pollen/ovule ratio ($t = 1.25$, $df = 18$, $P = 0.24$).

Self-compatibility

There was no significant difference in seed number of paired flowers subjected to hand selfing and hand outcrossing in the low altitude population in both years ($t = 0.57$, $df = 29$, $P = 0.58$ in 2008 and $t = 0.55$, $df = 29$, $P = 0.64$ in 2009) and in the high altitude population in 2009 ($t = 0.01$, $df = 29$, $P = 0.99$) (fig. 2), implying that *M. stylophorus* is fully self-compatible.

Capacity for autonomous selfing and apomixis

None of the flowers netted after emasculation and those without emasculation produced any seeds in the two popu-

Table 1 – Two-way ANOVA on the magnitude of pollen limitation, with year and population as fixed factors.

| Source | Pollen limitation | | | |
|--------------------------|-------------------|-----|-------|--------|
| | Sum Squ. | df | F | Sig. |
| Year | 0.69 | 1 | 5.79 | 0.02 |
| Population | 0.23 | 1 | 1.92 | 0.17 |
| Year \times Population | 1.95 | 1 | 16.40 | < 0.01 |
| Total | 23.61 | 112 | | |

lations in both years, suggesting there was no apomixis or autonomous selfing occurring, and thus pollinators are necessary for seed production in this species.

Emasculated flowers produced as many seeds as the paired control flowers in both populations in both years (In the low altitude population: $t = 0.52$, $df = 29$, $P = 0.61$ in 2008 and $t = 0.33$, $df = 29$, $P = 0.75$ in 2009; In the high altitude population: $t = 0.46$, $df = 29$, $P = 0.68$ in 2008 and $t = 1.03$, $df = 29$, $P = 0.54$ in 2009;), indicating that emasculation did not affect pollinator visitation.

Pollen limitation

Compared to the control flowers, supplemental hand-outcrossing increased seed production in both populations in both years (for the low altitude population: $t = 4.29$, $df = 29$, $P < 0.01$ in 2008 and $t = 2.33$, $df = 29$, $P = 0.03$ in 2009; for the high altitude population: $t = 2.81$, $df = 29$, $P < 0.01$ in 2008 and $t = 2.64$, $df = 29$, $P = 0.02$), showing pollen limitation under natural conditions (fig. 3). The magnitude of pollen limitation was significantly different between years but not among populations (table 1), suggesting the wide occurrence and inter-annual fluctuations of pollen limitation of seed production of *M. stylophorus*.

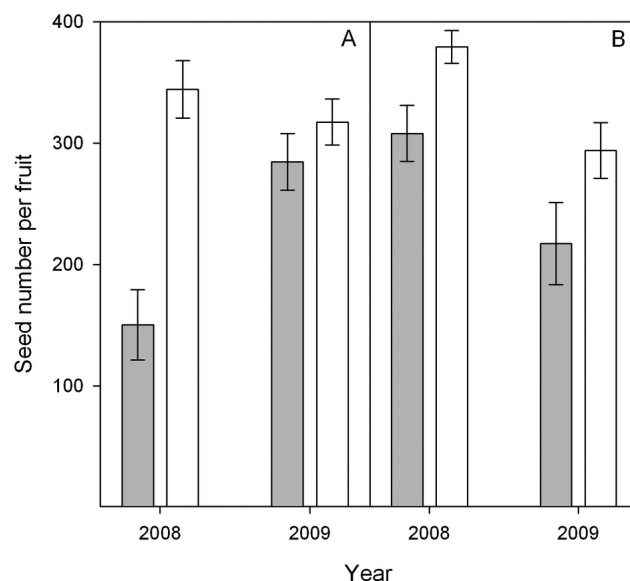


Figure 3 – Seed number of opposite flowers subjected to natural pollination (filled flowers) and supplemental pollination (open bars) in the Lulang population (A) and the Sejila population (B). Data is shown with mean \pm 1 SE.

Table 2 – Three-way ANOVA on the bumblebee visitation rate, with flower phase, population and year as fixed factors.

| Source | Sum Squ. | df | F | Sig. |
|----------------------------------|----------|-----|-------|--------|
| Flower phase | 2.02 | 1 | 11.55 | < 0.01 |
| Population | 3.99 | 1 | 22.82 | < 0.01 |
| Year | 4.41 | 1 | 25.26 | < 0.01 |
| Flower phase × Population | 0.63 | 1 | 3.59 | 0.06 |
| Flower phase × Year | 0.09 | 1 | 0.49 | 0.48 |
| Population × Year | 0.09 | 1 | 0.52 | 0.47 |
| Flower phase × Population × Year | 0.01 | 1 | 0.05 | 0.83 |
| Total | 370.38 | 439 | | |

Pollinator visitation rate

In ten days of observations in 2008 on the two populations (more than 70 hours in total for each population), bumblebees were found to be the most frequent visitors to *M. stylophorus* (fig. 1F). Bumblebees entered the flowers along the side of the corolla, with their back towards the center of the flowers (fig. 1F), where the anthers and stigmas are found, indicating that bumblebees were the potential pollinators of *M. stylophorus*. In the low altitude population, *Bombus richardsi* Reing and *B. sonani* Frison were the main pollinators, and *B. sonani* and *B. atrocinctus* Smith were the main pollinators in the high altitude population. However, in both populations, it was almost impossible to distinguish the bumblebee species while recording the visiting times of bumblebees, so the average visitation rates of the two bumblebee species were used.

In general, visitation rate was affected significantly by flower phase, year and population, but not by the interactions between any two factors (table 2). Bumblebees visited flowers in male phase more frequently than those in female phase, and the visitation rate in the low altitude population was much higher than in the high altitude population (table 2, fig. 4A & B).

DISCUSSION

Breeding system of *M. stylophorus*

Flowering development suggested a characteristic breeding system with combined dichogamy and herkogamy in *M. stylophorus*, which is common in alpine gentians (e.g. in Duan et al. 2005, Duan & Liu 2003, He & Liu 2004, Hou et al. 2009, Hou et al. 2008 and references therein). It is well known that the co-occurrence of dichogamy and herkogamy could separate pollen removal and deposition temporally and spatially, and further prevent autonomous selfing effectively. However, it is also possible that the co-occurrence of dichogamy and herkogamy could play an important role in reducing sexual interference (Barrett 2002, Duan et al. 2005, Lloyd & Webb 1986, Webb & Lloyd 1986). In the present study, the role of combined dichogamy and herkogamy was not examined, but this characteristic could prevent autonomous

selfing effectively in *M. stylophorus*, which was further indicated by the lack of seed production in the netted flowers without emasculation. Taking floral traits, pollen/ovule ratio and bagging experiments together, it might be concluded that *M. stylophorus* has a mixed mating system, in which geitonomous selfing might contribute to seed production to some degree because this species is self-compatible and bumblebees might visit more than one flower on one plant, and pollination agents are necessary for successful seed production of this plant. Therefore, future studies on mating pattern of using allozymes or microsatellites would be helpful in understanding the reasons for the low level of genetic diversity within populations of this plant (Ge et al. 2005).

Bumblebees are the dominant pollinators in the harsh environments, e.g. alpine and arctic (Heinrich 1975, Bergman et al. 1996, Bingham & Orthner 1998, Wesselingh et al. 2000). Our former studies also suggested bumblebees were the main pollinators of alpine plants in the QTP (Duan & Liu 2003, 2007, Duan et al. 2005, 2009, Hou et al. 2008). In the present study, bumblebees were found to be the only pollinators of *M. stylophorus*. Comparing the two populations, there was a trend of altitudinal decline in bumblebee visitation rate to both male and female flowers of *M. stylophorus*, which could result from the harsher environment in the higher altitude population. However, studies on more populations in both altitudes should be carried out in the future researches to confirm this altitudinal trend. Furthermore, the visitation rates of bumblebees to *M. stylophorus* were higher than those to other outcrossing gentians in the QTP, e.g. *Gentiana straminea* (He & Liu 2003, Duan et al. 2005). The possible reason could be the lower latitude in the Hengduan Mountains and the southeast corner of the QTP, where the two populations of *M. stylophorus* studied are located, compar-

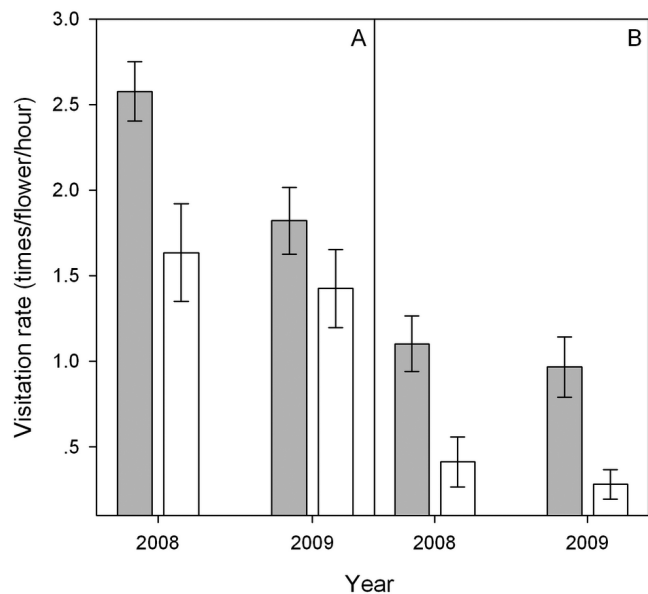


Figure 4 – Bumblebee visitation rates (visits/flower/hour) to male phase (filled bars) and female phase (open bars) flowers in the low altitude population (A) and the high altitude population (B). Data is shown with mean ± 1 SE, and result of comparisons is shown in table 2.

ing to the northeast of the QTP at higher latitude. Therefore, the more benign conditions in our study sites might result in the higher bumblebee visitation rates, although the number of bumblebee species did not increase.

Empirical evidence suggests that female reproductive success of plants decreases in small populations due to reduced pollinator services (e.g. Jacquemyn & Brys 2008, Brys et al. 2011), and thus selection for reproductive assurance would drive the evolution of autonomous selfing in small populations (Kalisz et al. 2004, Jacquemyn & Brys 2008, Brys et al. 2011). For *M. stylophorus*, habitat fragmentation induced by logging was severe during our field expeditions, and several large populations have been divided into isolated patches. Although we did not check the effects of population size on the pollinator services in *M. stylophorus*, pollen limitation could be induced by low levels of pollinator services due to reduced population size.

Implications for conservation

Although bumblebee pollination was considered to be an effective way to overcome the pollen limitation in alpine plants (Blionis & Vokou 2001, Bingham & Orthner 1998), seed production of *M. stylophorus* was still pollen limited in both populations in both years. Furthermore, population only had a minor effect on pollen limitation, indicating that pollen limitation might occur commonly throughout all the populations of *M. stylophorus*. Although severe pollen limitation of seed production could increase the threat to rare plants (Garg & Rao 1997), this is not the main reason for the endangerment of this species, because almost all control flowers in the two populations could produce seeds successfully in both years.

Strong genetic differentiation detected by ISSR indicated that almost no effective long-distance gene flow induced by pollen and/or seeds among the scattered populations of *M. stylophorus* (Ge et al. 2005), and this was further confirmed by the phylogeographical studies that almost no shared haplotype was found amongst 16 haplotypes in all the populations (Xue-Jun Ge, South China Botanical Garden, CAS, Guangzhou, China, pers. comm.). Therefore, considering the genetic diversity, all populations should be included for the effective *ex situ* and *in situ* conservation of *M. stylophorus*. However, *ex situ* conservation might be unpractical for two reasons. Firstly, it would be a huge and costly project to introduce individuals from all populations of *M. stylophorus* into a garden. Secondly, although seed collection is feasible theoretically for *ex situ* conservation, it seems unpractical because almost no seed germinated in laboratory experiments, which might contributed greatly to endangerment of *M. stylophorus* (unpublished data). We suggest *in situ* conservation is a more practical method in the present time, which protects the habitats of this species at the same time. As far as we know, the forest margins where this species occurs have already been severely destroyed by logging by the local people. Meanwhile, further researches on seed biology should be carried out to explore the *ex situ* conservation measures of this endangered species.

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