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SHORT COMMUNICATION

Reproductive biology of *Primula beesiana* (Primulaceae), an alpine species endemic to Southwest China

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Background and aims – *Primula beesiana* Forrest is a perennial alpine species endemic to southwest China, which is narrowly distributed in the eastern Himalaya and the Hengduan Mountains. The aim of this study is to elucidate the reproductive strategies of this heterostylous species in an alpine environment.
Methods – Floral scents were assessed and the pollinator assemblage and their foraging behaviour were recorded. In addition, the breeding system was investigated during 2004 and 2005 in populations from the Yulong Shan range, Lijiang County of Yunnan Province, China.
Key results – Limonene was the dominant floral scent compound, accounting for 41.2%, followed by α-pinene, diacetone alcohol and myrcene. Higher fruit and seed sets from inter-morph pollination were found compared to self, intra-morph and geitogamous pollination. Significant differences in seed set after self and open pollination were detected between flower morphs in both years. Two common insect species, the bumblebee *Bombus lucorum* and the butterfly *Hypolimnas misippus*, were the most important pollinators in the examined population, they visited the inflorescences 3.3 and 2.3 times per hour, respectively.
Conclusion – *beesiana* is an obligate outcrosser, but with some self-compatibility. Seed set of self-pollinated pins was higher than that of thrums in both years, which seems to be related to this partial self-compatibility, which is often ignored in distylous *Primula* species. Floral scent and petal colour provide signals for pollinator attraction, and visitation rates are high.

Key words – Alpine habitat, flower scents, heterostyly, reproductive success, pollination, *Primula beesiana*.

INTRODUCTION

Heterostyly is a form of sex differentiation that has evolved independently in at least 28 animal-pollinated angiosperm families (Barrett 2002). In heterostylous plants, populations are exhibiting two (distyly) or three (tristyly) flower morphs that differ in the height between positions of female and male reproductive organs (Barrett 2002). Heterostyly was first investigated in *Primula*, which appeared as the most distinctive feature in this genus: 91% of approximately 430 species of genus *Primula* are distylous (Richards 2002), and the pollination biology and mating system of *Primula* have been documented for several heterostylous species distributed in Europe, Japan and North America (e.g. Campbell et al. 1986, Miller et al. 1994, Washitani et al. 1994, Brys et al. 2004, Van Rossum et al. 2006). However, little is known about the pollination biology of *Primula* species at much higher altitudes (> 2700 m a.s.l.), especially for the species in the region of Himalaya and western China, which is regarded as the geographical origin and current centre of diversity for *Primula*.

Generally, the conditions in alpine environments are harsher than those in low altitude areas; they are characterized by high winds, low temperature, cloudiness, humidity and rapid weather changes. These factors have a negative effect on the frequency of insect visits (e.g. McCall & Primack 1992, Lázaro et al. 2008, 2013), and therefore might consequently affect reproductive fitness. Furthermore, in alpine conditions some plants change their reproductive system from obligate outcrossing to selfing by autonomous self-pollination via different strategies during anthesis (e.g. Kelso 1992, de Vos et al. 2012). Therefore, comprehensive pollination studies of alpine *Primula* in Himalaya would be expected to reveal some key reproductive strategies for adaptation to this extreme environment.

Primula beesiana Forrest is a perennial alpine species endemic to southwest China, growing in marshy mountain

meadows and on the borders of ditches and streams, at an altitude of 2700–3300 m (Hu & Kelso 1996). However, little is known about its pollination biology in the field. Here, we investigated the reproductive biology of *P. beesiana* over two flowering seasons to better understand the reproductive strategies of this heterostylous plant species in an alpine environment by a comprehensive investigation of (i) its floral scents as well as the pollinator assemblage and their foraging behavior and (ii) its breeding system by a series of hand pollination experiments.

MATERIALS AND METHODS

Study species and study site

Primula beesiana is a rosette-forming alpine perennial. The flowers of *P. beesiana* have the typical heterostylous morphology (Hu & Kelso 1996, Richards 2002). The petal colour is pink and the reflectance spectrum clearly showing a marked peak in the reflectance spectrum at 430 nm (fig. 1). It flowers from late May to mid-July. The flowers of both morphs open in the morning and the lifespan of a single flower is 6–9 days (Wu 2008). Anther dehiscence occurs 1–24 h after the opening of the petals. The stigma surface usually becomes wet, i.e. receptive to pollen, at the same time as the corolla starts to open, and it remains wet throughout anther dehiscence (Wu 2008).

The field study was conducted in a population of *P. beesiana* in the Yulong Shan range (27°00′15″N 100°10′70″E; 3020–3050 m a.s.l.), in Lijiang County of Yunnan Province, southwestern China, during the flowering seasons of 2004 and 2005. The climate at the study site during the rainy season (May to September) is foggy, cool, wet, and typically cloudy (data from Lijiang City Weather Bureau). The daily mean air temperature and humidity recorded at the meteorological station at Yunshanping in the Yulong Shan at the same altitude for the flowering season was approximately 12°C and 90%, respectively. Relative humidity was 25% in June 2004 but 15% in June 2005, indicating an exceptionally dry year.

Floral scent collection and analysis

Floral scents were collected using the dynamic headspace adsorption method (Chen et al. 2012) during daytime between

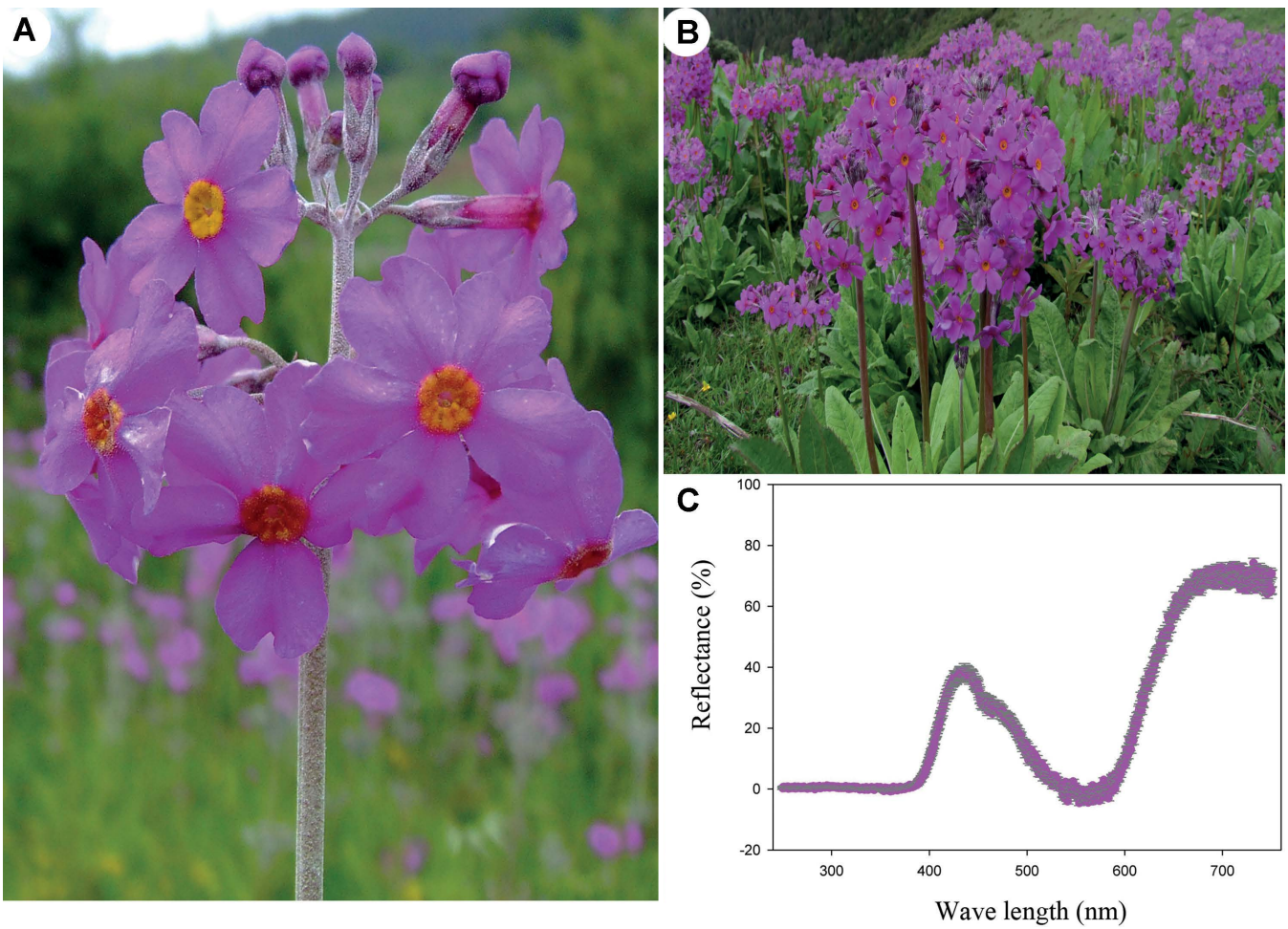

Figure 1 – Floral colour morphs of *Primula beesiana* and its associated reflection spectrum: A, an inflorescence of *P. beesiana*; B, flowering plants of *P. beesiana* and C, its associated reflection spectrum. The average spectral profiles of the purple petal colour are shown with their standard deviation (n = 20).

Table 1 – Average relative amounts (%) of floral scent compounds from *P. beesiana* using GC-MS analysis.
Listed are the Chemical Abstracts Service registry number (CAS), the retention time of the compound (*Rt*) in seconds.

No.	Compound	Rt	KI	CAS	Relative content (%)
1	Diacetone alcohol	6.69	1014.52	123-42-2	13.2
2	α-Pinene	10.01	929.5	80-56-8	16.4
3	Sabinene	11.71	970	3387-41-5	1.7
4	*β*-Pinene	11.83	972.86	127-91-3	1.9
5	3-Octanone	12.38	1005.50	106-68-3	3.4
6	Myrcene	12.54	989.76	123-35-3	9.8
7	Limonene	14.19	1029.05	123-35-3	41.2
8	Ocimene	15.08	1050.24	13877-91-3	4.1
9	Linalool	17.21	1100.83	78-70-6	1.6
10	Nonanal	17.41	1109.31	124-19-6	2.3
11	Decanal	29.90	1403	112-31-2	0.7
12	Isolongifolene	26.83	1405.02	1135-66-6	1.1
Total					97.2

12:00 h and 15:00 h, which corresponded to the time of effective pollinator activity. Three inflorescences with a total of 135 flowers of *P. beesiana* from three plants were treated to compare their emission levels (n=3). Following the protocol of Chen et al. (2012), newly opened inflorescences were enclosed in Tedlar bags (Dupont USA) and volatiles were drawn from the enclosures into cartridges containing the adsorbent Porapak Q (150 mg, mesh 60/80, Waters associates, Inc.) for 3 h using a pump (inlet flow rate 300 ml min^{-1}). Prior to use, the adsorbent cartridges were cleaned with 2 ml diethyl ether and dried with nitrogen gas. Trapped volatiles were eluted with 400 µl dichloromethane and concentrated to one-fifth of the original volume by a gentle stream of nitrogen. 720 ng of *n*-nonane was added to each sample for quantification, and the samples were stored at -20°C for subsequent analysis.

Extracts from the inflorescences were analyzed using an Agilent Technologies HP 6890 gas chromatograph, equipped with a HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness), and linked to a HP 5973 mass spectrometer. Helium was used as a carrier gas at a flow of 1 ml min^{-1}, and injector temperature was set to 250°C. Column temperature was 40°C and after injection, was increased to 250°C at a rate of 3°C min^{-1}. Compounds were identified by comparing mass spectra and retention times with values of reference compounds obtained from the National Institute of Standards and Technology (NIST, US Department of Commerce) Standard Reference Database (http://webbook.nist.gov/chemistry/) and a chemical suppliers' website (http://www.lookchem.com/). Kovats Index (KI) was calculated according to the formula as given in Chen et al. (2012): KI = 100n + 100(tx – tn)/(tn+1 – tn), where n is the number of carbon atoms in the n-alkane eluting immediately before the compound of interest.

Observations and collection of flower visitors

Insects visiting flowers of *P. beesiana* were observed in the field during peak flowering (12–18 June 2004 and 14–20 June 2005), and at the end of flowering (9–10 July 2005), in an area of approximately 5 × 5 m^2. Each observation day lasted from 07:30 h to 18:00 h. Once it started raining, field observations were stopped. To monitor whether night-flying insects visited *P. beesiana*, we also conducted observations from 20:00 h to 00:00 h on 16, 19, and 20 June 2004.

Flower visitor behaviour was observed and documented by photography. The visitation frequency of different visitors to flowers per inflorescence per hour and the duration of foraging on the flowers were recorded. Foraging insects were captured to be identified in the laboratory and examined for the presence of pollen by light microscopy**.** Voucher specimens of the insect visitors have been deposited in the Kunming Institute of Botany, CAS.

Pollination experiment

To investigate self-compatibility and effectiveness of possible modes of pollination, flowers from ten plants (fifteen plants for open pollination) of each morph at random were subjected to nine different treatments in mid-June of 2004 and 2005: (1) *self*: self-pollination within the same flower and bagged; (2) *geito*: geitonogamous pollination between flowers in the same individual and bagged; (3) *intra*: intramorph outcrossing between individuals of the same morph and bagged; (4) *inter*: inter-morph outcrossing and bagged; (5) *emasc*: emasculated but otherwise exposed to natural pollination; (6) *em+bag*: emasculated and bagged; (7) *em+net*: emasculated and netted; (8) *bag*: non-manipulated and bagged and (9) *open*: open pollination. All manipulations were conducted prior to anther dehiscence except for

Table 2 – Floral visitors of *Primula beesiana* in 2004 and 2005.

Insect species	Family	Foraging for	Voucher number
Pachliopta aristochiae	Papilionidae	Pollen, nectar	Wu20040101
Colias fieldii chinensis	Pieridae	?	Wu20040201, Wu20050201
Fabriciana adippe	Nymphalidae	?	Wu20040301-303, Wu20050301
Hypolimnas misippus	Nymphalidae	Pollen, nectar	Wu20040401-412, Wu20050401-417
Udara dilecta	Lycaenidae	?	Wu20050501-502
Bombus lucorum	Apidae	Pollen, nectar	Wu20040601-518, Wu20050601-520
Bombus sp.	Apidae	Pollen, nectar	Wu20040701-602
Cicindela sp.	Cicindelidae	Nectar or pollen	Wu20040801, Wu20050801-802
Rhizotrogus fraxinicola	Melolonthidae	Nectar or pollen	Wu20050902
Macroglossum sp.	Sphingidae	Nectar or pollen	Wu20041002

treatment *self* (these plants were self-pollinated at the time of anther dehiscence and then emasculated). Any previously opened flowers were removed from the plants, except for the *open* treatment. For treatment *bag*, flowers were bagged from bud to calyx closure stages, and then the bags were removed. Bags of thick waterproof paper were used to prevent pollination by both insects and wind, whereas nets of fine nylon fabric were used to prevent pollination by insects only. Capsules from all the treatments were harvested before dehiscence in late August and early September in 2004 and 2005. Fruit set was calculated as the percentage of flowers that developed into fruits and seed set was determined as the ratio of expanded seeds to ovules per fruit. The level of pollen limitation (PL) was also quantified for fruit set and for seed set, and for each morph and year by the formula: PL = 1 – naturally pollinated/hand pollinated (Larson & Barrett 2000).

Statistical analysis

All data was examined for normality and homogeneity of variance with a one sample Kolmogorov-Smirnov test. Prior to analysis, seed set for each plant was averaged. For fruit set, chi-square tests were used to detect global differences among all treatments per morph and per year, followed by pairwise tests for all possible treatment combinations. *T*-tests were used to compare fruit and seed set between morphs per treatment. Two-way ANOVA analysis was used to compare differences in seed set between morphs and years per treatment. Comparisons of seed set between treatments were further examined by Tukey's test after ANOVA. All statistical analyses were performed using R for Windows (R Development Core Team 2009).

RESULTS

Floral scents

In total twelve compounds were identified, which represented 97.2% of the total floral scent in *P. beesiana* (table 1). Among these detected volatiles, limonene was the major compound with 41.2%. In addition, α-pinene, diacetone alcohol and myrcene were detected, accounting for 16.4%, 13.2% and 9.8%, respectively.

Insect visitors and their behaviour

A total of ten species of insects were found to be active in the population, including five butterfly species, two bumblebee species, two beetle species and one hawkmoth (table 2). In clear weather, bumblebees began to visit and forage on *P. beesiana* and adjacent plants at about 08:00 h, up to 17:30–18:30 h, and had an activity peak at 12:00–13:00 h. Butterflies appeared at 09:00 h, through 18:00 h, with an activity peak at 14:00–15:00 h (fig. 2). The main pollinators were sensitive to weather changes, and they disappeared quickly when it began to rain and reappeared as soon as it cleared up.

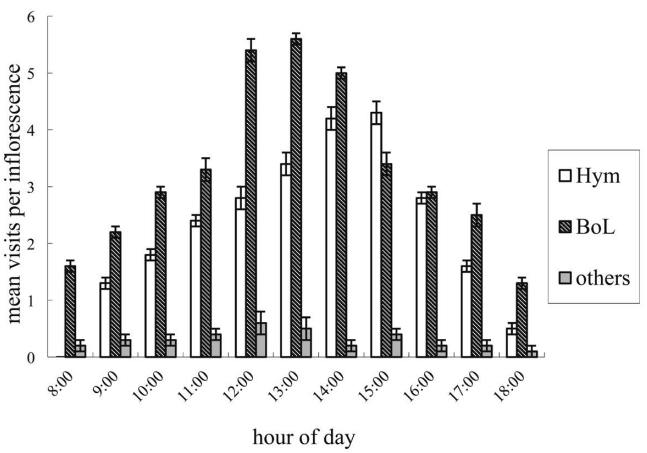

Figure 2 – Insect visitation rates (visits/inflorescence/hour) at peak flowering. Hym= *Hypolimnas misippus*, BoL= *Bombus lucorum*, others= the other eight visitor species.

Table 3 – **Fruit set and seed set (means ± s.e.) after different treatments in 2004 and 2005.**
P-values indicate the significance of the t-tests on the difference between pin and thrum within each treatment, treatments sharing the same superscript letter are not significantly different from each other in the same year and morph. *self*: self-pollination; *geito*: geitonogamous pollination; *intra*: intra-morph outcrossing; *inter*: inter-morph outcrossing; *emasc*: emasculated; *em+bag*: emasculated and bagged; *em+net*: emasculated and netted; *bag*: non-manipulated and bagged and *open*: open pollination. PL = pollen limitation = 1–open/inter.

Treatment	N flowers		N fruits (fruit set, %)			Seed set (%)		
	pin	thrum	pin	thrum	P-value	pin	thrum	P-value
2004								
self	153	166	17 (11.1%) [b]	6 (3.6%) [b]	0.0287	8.3 [b] ±1.0	2.1 [b] ±0.9	0.0007
geito	142	127	16 (11.3%) [b]	7 (5.5%) [b]	0.1829	7.9 [b] ±1.3	3.6 [b] ±1.0	0.0260
intra	144	132	21 (14.6%) [b]	13 (9.8%) [b]	0.3813	9.5 [b] ±0.3	4.9 [b] ±1.1	0.0012
inter	114	124	102 (89.4%) [c]	98 (79.0%) [c]	0.5819	62.3 [c] ±1.2	63.1 [c] ±1.4	0.4874
emasc	256	272	186 (72.6%) [c]	201 (73.9%) [c]	0.9526	56.5 [c] ±1.6	58.2 [c] ±1.2	0.2926
em+bag	283	304	0 [a]	0 [a]	1	0 [a]	0 [a]	1
em+net	326	297	0 [a]	0 [a]	1	0 [a]	0 [a]	1
bag	339	358	0 [a]	0 [a]	1	0 [a]	0 [a]	1
open	1104	1071	820 (74.2%) [c]	801 (74.8%) [c]	0.9423	59.0 [c] ±0.5	60.1 [c] ±0.5	0.0431
PL			0.17	0.05		0.05	0.05	
2005								
self	168	159	12 (7.1%) [b]	5 (3.1%) [b]	0.1945	6.8 [b] ±1.2	2.0 [b] ±0.8	0.0043
geito	153	166	14 (9.15%) [b]	12 (7.2%) [b]	0.7090	8.5 [b] ±1.4	6.5 [b] ±1.1	0.3163
intra	135	141	19 (14.0%) [b]	14 (9.9%) [b]	0.4494	8.3 [b] ±0.3	4.2 [b] ±0.7	0.0003
inter	128	116	113 (88.3%) [c]	102 (87.9%) [c]	1	62.1 [c] ±1.3	64.6 [c] ±1.4	0.1291
emasc	216	198	185 (85.6%) [c]	172 (86.9%) [c]	0.9796	64.3 [c] ±1.4	65.3 [c] ±1.2	0.4240
em+bag	224	237	0 [a]	0 [a]	1	0 [a]	0 [a]	1
em+net	267	281	0 [a]	0 [a]	1	0 [a]	0 [a]	1
bag	318	294	0 [a]	0 [a]	1	0 [a]	0 [a]	1
open	1136	1113	956 (84.1%) [c]	947 (85.1%) [c]	0.8844	66.7 [c] ±0.4	67.8 [c] ±0.4	0.0136
PL			0.05	0.03		-0.07	-0.05	

The insects that most frequently visited the flowers were *Hypolimnas misippus* and *Bombus lucorum* with 2.28±1.4 and 3.28±1.47 visits per inflorescence per hour, respectively (fig. 2). Pollen grains of *P. beesiana* were found attached to the proboscis of *H. misippus* and *B. lucorum*. Although the other eight species of insects visited the flowers as well, they visited flowers at a very low rate and no or few pollen grains were observed on their bodies. Therefore, *H. misippus* and *B. lucorum* were determined to be the most effective pollinators of *P. beesiana* in this habitat. No nocturnal insects were visiting *P. beesiana* during the night observation periods in June 2004.

Breeding system

The overall chi-square tests for all the treatments per morph per year revealed highly significant differences among treatments (X^2 values between 363.4 and 448.6, df = 8, all $P < 0.0001$). Subsequent pairwise comparisons of fruit set among treatments (table 3) showed that fruit set was not significantly different between *self*, *geito* and *intra*. The treatments *self*, *geito*, and *intra* produced fewer fruits in both years than the *open* treatment. The treatments *inter* and *emasc* produced as many fruits as the control flowers (*open*) in both years, meaning that hand outcrossing did not increase fruit production compared with natural pollination, and therefore pollen limitation was not significant. The treatments *emasc+bag, emasc+net* and *bag* did not produce any fruits or seeds in 2004 or 2005.

The naturally pollinated treatments (*emasc* and *open*) all produced on average more than 78 seeds per capsule (table 3), and together with *inter*, they had significantly higher seed sets than the *self*, *geito* and *intra* treatments. The seed set of *emasc* and *open* did not vary significantly between flower morphs, but was significantly higher in 2005 (table 4), whereas the seed set of treatment *inter* was not significantly affected by morph or year. For the treatment *self*, seed set was significantly higher in pins compared to thrums across years, and this was also true for *intra* in both years and *geito* in 2004 (table 3). In contrast, the thrums tended to have higher seed set in outcrossing treatments, but this was not significant, except in open pollination.

DISCUSSION

Volatile compounds and effective pollinators in *Primula beesiana*

To our knowledge, floral scents from only three species of *Primula* have been analyzed: *P. elatior*, *P. farinosa* and *P. spectabilis* (Gaskett et al. 2005, Vitalini et al. 2011). Our results revealed that limonene was the major compound in *P. beesiana*, accounting for 41% of the floral scent, followed by α-pinene, diacetone alcohol and myrcene. Limonene was

Table 4 – Two-way ANOVA on seed set with morph and year as fixed factors.

Treatment	Source	Sum squ.	Df	F-value	P-value
	Morph	585.2	1	31.54	<0.0001
self	Year	11	1	0.327	0.5710
	Morph×Year	605.27	3	10.603	<0.0001
	Morph	89	1	2.759	0.1050
inter	Year	9.1	1	0.264	0.6100
	Morph×Year	111.54	3	1.1128	0.3566
	Morph	62.4	1	1.036	0.3150
emasc	Year	1060	1	31.25	<0.0001
	Morph×Year	1124.8	3	11.021	<0.0001
	Morph	79.1	1	2.224	0.1420
open	Year	1648.9	1	234	<0.0001
	Morph×Year	1715.4	3	94.407	<0.0001

also detected in *P. elatior* as the dominant volatile compound (Gaskett et al. 2005). Monoterpenes such as limonene, myrcene and α-pinene are the most common compounds in floral scents in angiosperms, especially for plant species with diverse pollinating insect taxa including bees, Lepidoptera, beetles, and fly species (e.g., Knudsen et al. 1993, Knudsen 2002 and references therein).

Our field observations of pollinators of *P. beesiana* supported this hypothesis, as insects from eight families were observed visiting the flowers. However, although most flowers are visited by a diversity of insects, only a few of these are actually effective pollinators, and hence only the bumblebees (especially *B. lucorum*) and butterflies (mostly *H. misippus*) were confirmed as the predominant pollinators of *P. beesiana.* Bumblebees and butterflies have been frequently reported as important pollinators of plant species in other alpine zones (e.g. Arroyo et al. 1982, Levesque & Burger 1982, Bingham & Orthner 1998). Pollinator investigation on two other *Primula* species, *P. parryi* and *P. angustifolia*, which are distributed at higher altitudes (approx. 3700 m a.s.l.) than *P. beesiana*, showed that bumblebees were the most abundant flower visitors (Miller et al. 1994).

Breeding system

The seed set of inter-morph crosses (*inter, emasc* and *open*) was high, whereas it was very low in self- or intra-morph crosses (*self, geito, or intra*) was very low, demonstrating that *P. beesiana* is an obligately outcrossing species. Therefore, these results indicate that the intra-morph cross is illegitimate in *P. beesiana*, and that it can be considered as a functionally heterostylous plant species, characterized by a self-incompatibility system. These reproductive characteristics are consistent with their floral morphology, and it is thought to have evolved as a means of avoiding inbreeding to ensure the effective exchange of pollen in disassortative mating (Barrett 1992, Brys et al. 2004).

Hand selfing of pin and thrum flowers yielded some seeds, indicating that self-incompatibility is not complete. Furthermore, more self- or illegitimate fertilization occurred in the pin morph of *P. beesiana*, suggesting that self-incompatibility is stronger in the thrum morph than in the pin morph. This is congruent with observations in *P. veris*, *P. merrilliana* and *P. mistassinica*, which showed a higher self-compatibility in pin × pin crosses (Wedderburn & Richards 1992, Chen 2009). Although the reason for this difference in success rates of self-fertilization between the two morphs is not revealed by this study, a thrum flower could more easily receive pollen grains of same flower on its stigma, thus the low level of self-compatibility in the thrum morph suggests a mechanism of avoiding inbreeding. A potential reproductive advantage of higher partial self-compatibility of the pin morph is an interesting topic that needs to be examined further.

Hand outcross pollination did not increase seed set compared to open pollination, suggesting that no pollen limitation occurred in *P. beesiana* field populations, which is in contrast with the strong pollen limitation often found in other *Primula* species (Brys et al. 2007, Fisogni et al. 2011). This is likely due to the favourable weather during the flowering period of *P. beesiana*, which was often dry and clear, allowing pollinator activity. It should be pointed out that rainy periods generally overlap with the flowering time in *P. beesiana*. It is clear that there exists spatial and temporal variation in the links between plants and pollinators (e.g. Petanidou et al. 2008, Dupont et al. 2009). Although only two insect species, *H. misippus* and *B. lucorum*, were effective pollinators of *P. beesiana*, the pollinator visitation rate was higher than other *Primula* species (Fisogni et al. 2011, Chen 2009), providing an efficient pollination service. Meanwhile, the high-density patches of flowering plants may attract more pollinators, which decrease the level of pollen limitation. Moreover, the flowering time of *P. beesiana* lasts almost three months, and the lifespan of single flower is more than seven days,

which allows the stigma enough time to receive legitimate pollen grains carried by pollinators. It would be interesting to revisit the population of *P. beesiana* and observe the pollinator assemblage again, in order to test the stability of pollinator services, since habitat disturbance has become a bigger problem than ten years ago.

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