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Comparative study of pollination biology of two closely related alpine *Primula* species, namely *Primula beesiana* and *P. bulleyana* (Primulaceae)

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Abstract The comparative pollination biology of a population of *Primula beesiana*, a population of *P. bulleyana*, and an overlapping population of these two species in Yulong Shan, Yunnan Province, China, was studied in 2004 and 2005. The results indicate that both *P. beesiana* and *P. bulleyana* are typical heterostylous and obligate outcrossing species; the main pollinators of the two species were bees and butterflies. At the sites of all three populations, the main pollinating visitors of the two species showed preference for one of the two species, resulting in the ethological isolation of the two species by the pollinators. This ethological isolation contributes to the reproductive isolation of the two species, which supports the hypothesis that *P. beesiana* and *P. bulleyana* are two distinct species. The reproductive isolation of the two species (ethological isolation) is probably an important mechanism in maintaining species boundaries in the genus and has contributed to the species diversification of *Primula* in the area. In addition, gene exchanges between *P. beesiana* and *P. bulleyana* has occurred to some extent in the overlapping population, but whether natural hybridization has contributed to species diversification in *Primula* remains to be determined.

Key words pollination biology, *Primula beesiana*, *Primula bulleyana*, reproductive isolation.

Among flowering plants, biological diversity is a direct consequence of speciation and the evolution of reproductive isolation is central to the speciation process (Kay, 2006). The crucial step in the separation of two populations of a sexually reproducing species into two new species (i.e. speciation) is the acquisition of reproductive isolation between them. Such isolation ensures that the populations can evolve independently of each other and so end up with radically different genotypes and phenotypes (Charlesworth & Charlesworth, 2000). Understanding the types of barriers that contribute to reproductive isolation will help elucidate the conditions under which speciation is likely to occur and the role of natural selection in speciation (Charlesworth & Charlesworth, 2000; Kay, 2006). Previous studies have shown that much of our knowledge of isolating mechanisms comes from cases in which the taxa are currently found in at least partial sympatry and that most of the taxa are incipient or very closely related species (Hodges & Arnold, 1994; Melendez-Ackerman et al., 1997; Fulton & Hodges, 1999; Ramsey et al., 2003; Kay, 2006; Martin & Willis, 2007; Tang et al., 2007; Yang et al., 2007). Therefore, pollination biology on sympatric or partially sympatric closely related species may provide the most relevant data on the necessary conditions for the speciation of a clade.

Primula is one of the largest and most widespread genera in Primulaceae. Approximately 430 species in this genus are distributed throughout the moister and cooler regions of the Northern Hemisphere (Richards, 2002). The region of the Himalayas and western China has the greatest concentration of species and the greatest diversity of *Primula*, with over half of all *Primula* species found in, and most restricted to, this region. The Sino-Himalayan region is therefore regarded as the geographical origin and diversity distributional center of the genus (Wedderburn & Richards, 1992; Richards, 1993, 2002; Arnold & Richards, 1998).

The reproductive biology of the genus *Primula* (Primulaceae) first became known through the investigations of Darwin (1877) on distyly. Since then, to study distyly and its relationship with *Primula* reproduction, the pollination biology and mating systems of some species have been well studied, but most of these species are in Europe, Japan, and North America (Ornduff, 1979, 1980; Schou, 1983; Campbell et al., 1986; Miller et al., 1994; Washitani et al., 1994a, 1994b; Matsumura & Washitani, 2000; Brys et al., 2004). The pollination biology and ecology of alpine *Primula* species, especially in the region of the Himalayas and western China, are little known.

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Primula beesiana Forrest and *P. bulleyana* Forrest are two plants belonging to the section Proliferae in *Primula*. Both plants are distributed in the Lijiang range of Yunnan Province and the Muli area of Sichuan Province, China (Chen & Hu, 1990; Hu & Kelso, 1996). Although both plants are distributed in the same area, there is little overlap of the distributions of the two plants in the field; we have only found one overlapping population. The two plants have the same ecological conditions of growth, being found in marshy mountain meadows and on the sides of ditches and streams at 2500–3300 m above sea level (a.s.l.). *Primula beesiana* and *P. bulleyana* are structurally very similar, with the only distinguishing characters of the two plants being flower color and calyx segments. In *P. beesiana*, the flowers are rose-carmine and yellow-eyed with an orange tube, and the sepal-lobes are sharp; however, in *P. bulleyana*, the flowers are red in bud and deep orange when mature, and the sepal-lobes are awl-like. In the dried state, when the color of the flowers may be destroyed, the two plants can readily be confused. In addition, the two plants are interfertile in the garden (Chen & Hu, 1990; Richards, 1993, 2002). Based on their distribution, morphological similarity, and interfertility in cultivation, scholars had different ideas about their classification. Some regarded the two plants as two distinct species (Smith & Fletcher, 1941; Chen & Hu, 1990; Hu & Kelso, 1996), but some treated *P. beesiana* as a subspecies of *P. bulleyana* (Richards, 1993, 2002). Therefore, a study of the comparative pollination biology of the two plants may be helpful in understanding the relationship between the two plants and provide insights into the origins of species diversity of *Primula* in this area.

In the present study, the pollination biology of a population of *P. beesiana*, a population of *P. bulleyan*, and an overlapping population of these two species was investigated over a 2-year period. We conducted detailed comparative observations of pollination biology and tested the breeding system of the two species, focusing on the following four questions: (i) are these two species obligate outcrossing species; (ii) what are the main pollinators of these two species; (iii) do the main pollinators prefer to visit one of the two species; and (iv) what reproductive isolation mechanisms are responsible for the two species?

1 Material and Methods

1.1 Study plant and study site

Both *Primula beesiana* and *P. bulleyana* are perennial herbs. The scapes are 20–35 cm long, elongated to 70 cm in fruit; umbels 2–8, superimposed, each whorl 8–16 flowered. Flower buds bloom from the basal umbel to the distal umbel sequentially. Flowers are heterostylous, the populations consisting of two types of plants that differ in their floral morphology. One type, called the long-styled or pin morph, bears flowers with a long style and short stamens; the other, called the short-styled or thrum morph, bears flowers with short styles and long stamens.

Field studies were conducted in the Yulong Shan range, in Lijiang, Yunnan Province, southwest China, during the flowering seasons of 2004 and 2005. The location of the *P. beesiana* population was near Weihai village (27°00′15.0″ N, 100°10′70.7″ E; 3020–3050 m a.s.l.). In order to avoid the destruction of plants by grazing, field experiments were performed in a south-facing disused agricultural garden surrounded by a short wooden fence. At this site, *P. beesiana* was the dominant species, with thousands of individuals. The *P. bulleyana* population was located in a meadow by the Heishui river (27°01′35.6″ N, 100°10′34.7″ E; 3100 m a.s.l.), with individuals growing alongside a stream. At this site, *P. bulleyana* was also the dominant plant, growing in association with red-flowered *Primula poissonii* and other species. The overlapping population was located at a distance of approximately 1 km from the *P. bulleyana* population (27°01′53.1″ N, 100°10′21.7″ E; 2950 m a.s.l). At this site, individual plants of *P. beesiana* and *P. bulleyana* grew in groups of each species. During the rainy season, from May to September, the climate of the Yulong Shan at altitudes of 2950–3100 m a.s.l. is foggy, cool, and wet, with most days cloudy. Daily mean air temperatures and humidity for the flowering season, recorded at the meteorological station at Yunsanping in Yulong Shan at a similar altitude, were approximately 12°C and 90%, respectively.

1.2 Observations of floral phenology

In the flowering seasons of 2004 and 2005, 10 inflorescences of each morph in these three populations were inspected each day. At each inspection, newly opened and newly faded flowers were marked, and floral morphological changes, flowering period, flower lifespan, anther dehiscence, and stigmatic status recorded. To determine the floral morphological traits in the two plants, the stigma and anther heights (maximum height from the base of the ovary), corolla diameter, corolla tube diameter at the mouth, relative anther–stigma distance, and corolla tube length were measured to ±0.1 mm using a Vernier calipers for 100 randomly selected flowers from 100 individuals of each morph in the *P. beesiana* and *P. bulleyana* populations in 2004.

In the *P. beesiana* and *P. bulleyana* populations, the spatial structure and the frequency of pins and thrums in the population were determined by counting all flowering plants in 20 random 1-m^2 plots during the flowering season of 2004. To determine whether the morphs were randomly distributed, clumped, or segregated, the nearest-neighbor analysis of Pielou (1961) was used: flowering plants were taken at random in these two populations, their morphs and the morph of the nearest neighbor were determined, and the data analyzed by a 2 × 2 contingency table.

1.3 Pollen:ovule ratio

The pollen:ovule ratios (P/O) of the two plants were estimated according to the methods of Cruden (1976) and Dafni (1992). A total of 21 flowers from 21 different individuals of each morph in either species were chosen at random, the number of pollen grains and ovules in each flower determined, and the mean±SD calculated.

1.4 Observations and collection of flower visitors

Flower visitors to the two plants and their adjacent plants at each site were observed in the field over the peak of flowering, in an area of approximately 25 × 25 m. Observations were performed from 07:30 h in the morning to 18:00 h in the evening under different weather conditions. Observation periods were divided into 15–30-min intervals. Many previous studies have stated that night-flying insects are important in the pollination system of *Primula* (Darwin, 1877; Lubbock, 1905; Woodell, 1960). To monitor whether night-flying insects visited the plants of the two species, we conducted observations from 20:00 to 00:00 h on 16, 19, and 20 June 2004 at the *P. beesiana* population and on 18, 19, and 21 June 2005 at the *P. bulleyana* population under different weather conditions. The behavior of flower visitors was observed and documented by photography. The visitation frequency of different visitors to flowers per inflorescence per hour was observed and recorded. The captured insect visitors were brought to the laboratory for identification and to examine them for the presence of pollen under an optical microscope. Voucher specimens of the insect visitors have been deposited in the Kunming Institute of Botany, Chinese Academy of Sciences.

1.5 Breeding system experiments

To investigate the breeding system and the effectiveness of insect visitors in pollen transfer, within each morph eight experimental treatments of 10 plants, each at random, and an open-pollinated treatment of 15 plants in the *P. beesiana* and *P. bulleyana* populations were established in mid-June 2004 and 2005. The treatments were as follows: (i) Treatment 1, emasculated and unbagged; (ii) Treatment 2, emasculated and bagged; (iii) Treatment 3, emasculated and netted; (iv) Treatment 4, hand pollinated and bagged (autogamy); (v) Treatment 5, hand pollinated and bagged (geitonogamy); (vi) Treatment 6, non-manipulated and bagged; (vii) Treatment 7, intramorphs crossed and bagged; (viii) Treatment 8, inter-morphs crossed and bagged; and (ix) Treatment 9, open pollination. All manipulations were conducted prior to anther dehiscence, with the exception of the selfing treatment (Treatment 4; these plants were self-pollinated at the time of anther dehiscence and then emasculated). The early opened flowers (anther dehisced flowers) were removed from the plants, except for the open-pollinated treatment. For Treatment 6 (non-manipulated and bagged), flowers were bagged from bud to calyx closure stages and the bags were then removed. Bags were used to prevent pollination by insects and air-disseminated pollen, whereas nets served only to prevent insects from visiting the flowers.

1.6 Statistical analysis

Differences in mean values of morphological parameters, pollen grain number, ovule number, and pollen:ovule ratios between the two species were tested by one-way ANOVA for both morphs of either species. All tests were performed using SPSS version 13.0 (SPSS, Chicago, IL, USA).

2 Results

2.1 Floral phenology of *Primula beesiana* and *P. bulleyana*

Primula beesiana and *P. bulleyana* both flowered from late May until mid-July; in the Yulong Shan range, peak flowering occurred between mid-June and early July. The flowers of pin morph and thrum morph opened at almost the same time in the same population of the two species. The lifespan of a single flower in both morphs of the two species (from the petals opening to stamens and petals being lost) was 6–9 days. At the peak of flowering, there were usually two whorls of umbels in the flower (10–30 flowers) in both species. Anther dehiscence usually occurred 1–24 h after the opening of the petals. It was also observed that the anthers of some flowers dehisced before the opening of the corolla in both morphs of the two species. The stigma surface usually started to become wet at the same time as the corolla started to open and the stigma remained wet throughout anther dehiscence in both species.

2.2 Floral traits of *P. beesiana* and *P. bulleyana*

Parameters describing the floral morphology of the two species are presented in Table 1. In each species,

Table 1 Floral morphological traits of *Primula beesiana* and *P. bulleyana*

Species	Morph	Corolla diameter (mm)	Corolla tube length (mm)	Stigma heights (mm)	Anther height (mm)	Stigma–anther distance (mm)	Corolla tube diameter at the mouth (mm)
P. beesiana	Pin	21.7 ± 2.0	12.3 ± 1.0	11.9 ± 1.0	7.2 ± 0.9	4.7 ± 0.4	1.5 ± 0.4
	Thrum	21.9 ± 2.0	12.6 ± 1.0	7.4 ± 0.9	12.0 ± 1.0	4.6 ± 0.3	1.9 ± 0.4
P. bulleyana	Pin	22.1 ± 1.3	12.4 ± 0.9	11.3 ± 0.7	7.0 ± 0.6	4.3 ± 0.4	1.4 ± 0.3
	Thrum	22.5 ± 0.9	13.1 ± 1.1	7.6 ± 0.6	11.5 ± 0.9	3.9 ± 0.7	1.9 ± 0.4

Values are means ± s.d. (unit: mm)

the corolla diameter and corolla tube length of pins and thrums did not differ significantly, but the corolla tube diameter at the mouth of thrums was significantly larger than that of pins (*P. beesiana* $F = 62.5$, $P < 0.001$; *P. bulleyana* $F = 116.1$, $P < 0.001$). This is because the anthers of thrums are borne near the mouth of the corolla tube, whereas the anthers of pins are borne near the middle of the corolla tube. The stigma and anther height differed significantly in the two morphs (stigma height: *P. beesiana* $F = 1251.0$, $P < 0.001$; *P. bulleyana* $F = 1658.2$, $P < 0.001$; and anther height: *P. beesiana* $F = 1354.4$, $P < 0.001$; *P. bulleyana* $F = 1509.7$, $P < 0.001$). In both species, the relative stigma–anther distance showed that the stigma position roughly coincided with the anther position of the opposite morph. The flower color of pins and thrums was the same within each species, but the rose-carmine color of *P. beesiana* flowers was significantly different from the deep orange color of the flowers of *P. bulleyana*.

In 20 random 1-m^2 plots of the *P. beesiana* population, of 262 flowering individuals, 136 plants were pins and 126 plants were thrums. The ratio of the two morphs was approximately 1:1 in the population. Nearest-neighbour analysis also gave the same result. Of 110 randomly selected plants, 47.3% were pins and 52.7% were thrums. Of these, 23 pin plants had a same morph individual as their nearest neighbor and 30 thrums had another thrum individual as their nearest neighbor ($\chi^2 = 0.054 < \chi^2{}_{0.05,1} = 3.84$; $P\ 0.05$, coefficient of segregation $S = -0.05$). In the *P. bulleyana* population, of 235 flowering individuals, 114 plants were pins and 121 plants were thrums. The ratio of the two morphs was also approximately 1:1 in the population. Nearest-neighbour analysis also yielded the same result. Of 100 randomly selected plants, 46.0% were pins and 54.0% were thrums. Of these, 22 pin individuals had a plant of the same morph as their nearest neighbor, and 28 thrums had another thrum individual as nearest neighbor ($\chi^2 = 0.028 < \chi^2{}_{0.05,1} = 3.84$; $P > 0.05$, $S = -0.003$). From these data it can be concluded that the two morphs are randomly distributed in both populations.

2.3 The P/O ratio of *P. beesiana* and *P. bulleyana*

The numbers of pollen grains, ovule numbers per flower and the P/O ratios of the two species are given in Table 2. In both species, the number of pollen grains in pin flowers was significantly greater than that in thrum flowers (*P. beesiana* $F = 134.101$, $P < 0.001$; *P. bulleyana* $F = 77.283$, $P < 0.001$), but the number of ovules per flower did not differ between the two morphs (*P. beesiana* $F = 0.015$, $P = 0.903$; *P. bulleyana* $F = 0.064$, $P = 0.802$). The P/O ratio in pin flowers was significantly higher than that in thrum flowers (*P. beesiana* $F = 86.777$, $P < 0.001$; *P. bulleyana* $F = 52.726$, $P < 0.001$).

2.4 Insect visitors and their behavior

2.4.1 *Primula beesiana* population site Based on our observations and the collection of foraging insects at the site during the two flowering seasons (2004 & 2005), a total of 10 species of insects (belonging to eight families and three orders) were found to be active (Table 3). These included five butterfly species, three bumblebee species, and two beetle species. *Hypolimnas misippus*, *Bombus richardsi*, and *Bombus lucorum* were the most common visitors in the community. *Hypolimnas misippus* and *B. lucorum* were the main visitors to *P. beesiana*, visiting at a high frequency on clear days (Fig. 1a). Examination of pollen loads showed that many

Table 2 Ovule and pollen numbers of *Primula beesiana* and *P. bulleyana*

Species	Morph	No. flowers	No. pollen grains/ flower (P)	No. ovules/ flower (O)	P/O ratio
P. beesiana	Pin	21	796 000 ± 109 000	139 ± 19	5820 ± 1100
	Thrum	21	434 000 ± 93 000	140 ± 23	3150 ± 720
P. bulleyana	Pin	21	682 000 ± 106 000	137 ± 10	5030 ± 980
	Thrum	21	438 000 ± 70 000	138 ± 12	3010 ± 600

Data are the mean ±s.d.

Table 3 Flower visitors and their rewards

Population	Insect*	*n*	Foraging object
Primula beesiana population	*Pachliopta aristochiae*	1	Pollen, nectar
	Colias fieldii chinensis	2	?
	Fabriciana adippe.	4	?
	Hypolimnas misippus	29	Pollen, nectar
	Udara dilecta	2	?
	Bombus richardsi	32	Pollen
	Bombus lucorum	38	Pollen, nectar
	Bombus sp.	2	Pollen, nectar
	Cicindela sp.	3	Nectar or pollen
	Rhizotrogus fraxinioola	2	Nectar or pollen
P. bulleyana population	*Aporia bieti*	27	Pollen, nectar
	Amegilla calceifera	18	Pollen, nectar
	Micrapis florae	23	Pollen, nectar
	Argyreus hyperbius	12	Pollen, nectar
	Byasa polyeuctes polyeuctes	1	Pollen, nectar
	Adiscus nigripennis	2	?
Overlapping population	*Hypolimnas misippus*	20	Pollen, nectar
	Bombus lucorum	17	Pollen, nectar
	Aporia bieti	18	Pollen, nectar
	Argyreus hyperbius	15	Pollen, nectar
	Fabriciana adippe	8	Pollen, nectar

*Insects were identified by Professor Li-Zhen WANG of Yunnan University (Kunming, China).

pollen grains of *P. beesiana* were attached to the proboscis of *H. misippus* and *B. lucorum*. These insects only visited *P. beesiana* and we did not observe their visitation to other plant species at the site. They were likely to be the most effective pollinators and preferred *P. beesiana* to other plant species in this habitat. *Bombus richardsi* was active in the community but, for the entire time of observation, we never saw one visiting *P. beesiana*. *Bombus richardsi* just visited the yellow-flowered plants: *Lotus corniculatus* var. *japonicus, Ranunculus sceleratus*, *Potentilla crenulata* and *Fragaria nilgerrensis*. The other seven species of insects, namely four butterfly species (*Pachliopta aristochiae*, *Colias fieldii chinensis, Fabriciana adippe*, and *Udara dilecta*), two beetle species (*Cicindela* sp. and *Rhizotrogus fraxinioola*), and one *Bombus* sp., were few in number and only one or a small number of them visited *P. beesiana* during the observation period across 2004 and 2005 (Table 3). Nocturnal insect pollination activity for *P. beesiana* was not observed from 20:00 to 00:00 hours on 16, 19, and 20 June 2004 under different weather conditions.

2.4.2 *Primula bulleyana* population site Six species of insects (belonging to six families and three orders) were found to be active at the site, namely three butterfly species, two bee species and one beetle species (Table 3). *Aporia bieti*, *Amegilla calceifera*, *Micrapis florae*, and *Argyreus hyperbius* were the most common visitors and, because all individuals surveyed carried *P. bulleyana* pollen, they were the main pollinators for *P. bulleyana*. On clear days they visited *P. bulleyana* at a high frequency (Fig. 1b) and showed a pollination preference for *P. bulleyana*. In the observations made across the 2 years, these insects were found to visit *P. bulleyana* only and they were not observed visiting plant species with flowers of other colors. Two other insects, namely *Byasa polyeuctes polyeuctes* and *Adiscus nigripennis*, were also found to visit *P. bulleyana*, but only one or

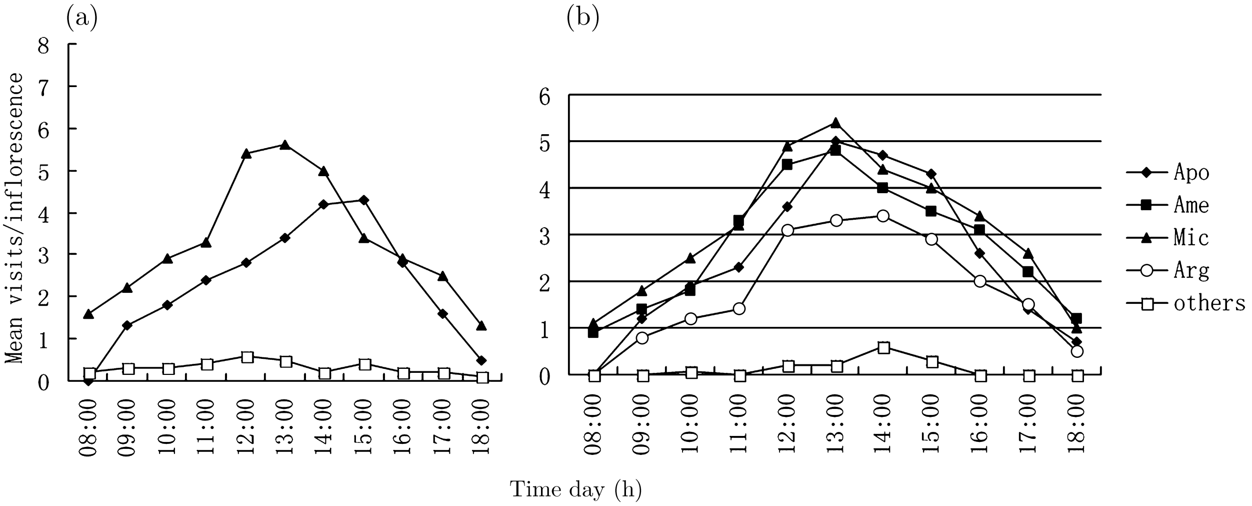

Fig. 1. Visitation frequency of insects at the peak of flowering. **a,** *Primula beesiana* population. (♦), *Hypolimnas misippus*; (▲), *Bombus lucorum*; (■), others. **b,** *Primula bulleyana* population. (■), *Amegilla calceifera*; (♦), *Aporia bieti*; (○), *Argyreus hyperbius*; (▲), *Micrapis florae*; (□), other visitors.

a small number of visits was recorded in the 2 years (Table 3). Nocturnal insect pollination activity for *P. bulleyana* was also not observed under different weather conditions.

2.4.3 Overlapping population site Five species of insects were found to be active at the site. *Hypolimnas misippus* and *Bombus lucorum* were the main pollinators for *P. beesiana*, whereas *Aporia bieti* and *Argyreus hyperbius* were the main pollinators for *P. bulleyana*. Thus, the main pollinators for the two plants in the population were very similar to those observed in separate *P. beesiana* and *P. bulleyana* populations, with each pollinating species showing a pollination preference for a particular species. However, there were shared pollinators in the population for the two plants. It was observed that a few *Fabriciana adippe* visited both species in the overlapping population.

The behavior of the main pollinators for *P. beesiana* and *P. bulleyana* was almost the same. Upon visiting the flowers of *P. beesiana* and *P. bulleyana*, the pollinators clung to the flower petals and, in the course of foraging, inserted their long proboscis into the tube of the corolla to gather nectar and/or pollen. The pollen at the mouth of the corolla tube (thrum) or in the middle of the corolla tube (pin) would then attach to their proboscis. When this forager visited another flower, it would leave or take more pollen, thus mediating pollen transfer between different flowers.

Weather had a significant effect on pollinator activity for both species in the alpine area. In clear weather, bees started becoming active in these populations at about 08:00 h and continued through to 17:30–18:30 h (Fig. 1a, b); peak activity was observed at 12:00–13:00 h. Butterflies appeared at 09:00 h and persisted through to 18:00 h, with a peak in activity at 14:00–15:00 h. However, the main pollinators were sensitive to weather changes. When it started to rain, the pollinators would disappear quickly and, when it began to clear, they would reappear in the population very soon.

2.5 Fruit-set and seed production

Fruit-set and seed production data from the breeding system experiments are presented in Table 4. The data suggest that the number of flowers of pins and thrums of the open-pollinated plants in each species is similar; there was no significant difference in the number of flowers produced by the two morphs in either species. In the breeding system experiments, only Treatments 1 (emasculated and unbagged), 8 (intermorphs crossed and bagged), and 9 (open pollination) produced fruit and seed normally in both species. Treatments 2 (emasculated and bagged), 3 (emasculated and netted), and 6 (non-manipulated and bagged) did not produce fruit. Some plants subjected to Treatments 4 (hand pollination and bagged; autogamy), 5 (hand pollination and bagged; geitonogamy), and 7 (intramorphs crossed and bagged) produced several fruits and seeds in both species, but the production ratios were very low; only a few flowers developed into fruits and the number of seeds produced per capsule was also very low.

Table 4 Fruit-set and seed production in breeding system experiments of *Primula beesiana* and *P. bulleyana*

Plant	Treatment*	No. plants		No. flowers		No. fruits (% fruits per flower)		Mean seeds per capsule	
		Pin	Thrum	Pin	Thrum	Pin	Thrum	Pin	Thrum
P. beesiana	1	20	20	472	470	371 (78.6%)	373 (79.3%)	83.8	86.0
	2	20	20	507	541	0	0	0	0
	3	20	20	593	578	0	0	0	0
	4	20	20	321	325	29 (9.0%)	11 (3.4%)	12.1	7.1
	5	20	20	295	293	30 (10.2%)	19 (6.4%)	13.8	10.4
	6	20	20	657	652	0	0	0	0
	7	20	20	279	273	40 (14.3%)	27 (9.9%)	12.5	8.5
	8	20	20	242	240	215 (88.8%)	200 (83.3%)	86.8	89.4
	9	30	30	2240	2184	1776 (79.2%)	1748 (80.0%)	87.2	89.6
P. bulleyana	1	20	20	510	498	409 (80.2%)	390 (78.3%)	86.2	84.4
	2	20	20	468	490	0	0	0	0
	3	20	20	503	476	0	0	0	0
	4	20	20	297	315	21 (7.0%)	15 (4.7%)	8.9	7.5
	5	20	20	312	298	36 (11.5%)	17 (5.7%)	11.2	5.4
	6	20	20	288	306	0	0	0	0
	7	20	20	263	252	36 (13.7%)	20 (7.9%)	10.4	8.2
	8	20	20	250	266	226 (90.4%)	82.1 (82.3%)	88.6	86.2
	9	30	30	2146	2230	1694 (78.9%)	1802 (80.8%)	88.9	86.8

*Treatment 1, emasculated and unbagged; Treatment 2, emasculated and bagged; Treatment 3, emasculated and netted; Treatment 4, hand pollination and bagged (autogamy); Treatment 5, hand pollination and bagged (geitonogamy); Treatment 6, non-manipulated and bagged; Treatment 7, intramorphs crossed and bagged; Treatment 8, inter-morphs crossed and bagged; and Treatment 9, open pollinated.

3 Discussion

The floral traits of *P. beesiana* and *P. bulleyana* indicate that the two plants are typical heterostylous species and are closed in floral morphology. In the breeding system experiments, flowers with self-pollination rarely set seeds, demonstrating that *P. beesiana* and *P. bulleyana* are obligate outcrossing species. In addition, the high P/O ratios in both morphs were also closer to those reported by Cruden (1976) for obligate outcrossing species (P/O = 5859.2) than that for facultative outcrossing species (P/O = 796.6). These reproductive characteristics are consistent with the floral morphology of *P. beesiana* and *P. bulleyana*: heterostylous flowers, which are associated with a sporophytic self-incompatibility system that prevents intramorph fertilization and encourages outcrossing between pins and thrums.

3.1 Pollinators for alpine *Primula* species

Observations in the field and the collection of visitors to the three populations suggested that *P. beesiana* and *P. bulleyana* had similar insect visitors, mainly bees and butterflies. There has been considerable argument about the pollinators of *Primula* species since the work of Darwin (1877). Based on previous studies (Woodell, 1960; Ornduff, 1980; Schou, 1983; Campbell et al., 1986; Miller et al., 1994; Washitani et al., 1994a), the present study and our observations of *P. poissonii* and *P. secundiflora* (Z.-K. Wu et al., unpubl. data, 2003), we conclude that Hymenopterans (*Bombus* spp.) and Lepidopterans (butterfly spp.) are important pollinators for most primulas, especially for alpine species, because their long proboscis can easily reach the nectar at the base of a long corolla tube in primulas. When these insects forage in different flowers, pollen transfer occurs between different flowers. Studies in other alpine zones have also shown that Hymenopterans and Lepidopterans are frequently important pollinators at high elevations (Arroyo et al., 1982; Levesque & Burger, 1982; Miller et al., 1994).

3.2 Reproductive isolation and its systematic implication

Insect-pollinated flowers have some means by which they attract insects: bright colors and strong scents are two of the most obvious (Woodell, 1960). In the case of *P. beesiana* and *P. bulleyana*, although they had similar main visitors (i.e. bees and butterflies), color probably played an important role in pollinator selection. In all three populations, the main visitors to *P. beesiana* and *P. bulleyana* could apparently detect the plants by color and discriminated strongly between the plants. At the *P. beesiana* population site, the main visitors to *P. beesiana* would not visit other colored species blooming simultaneously; at the *P. bulleyana* population site, the main pollinators of *P. bulleyana* also showed a pollinator preference for *P. bulleyana*; at the overlapping population site, although there were some shared visitors for the two species, the number of these visitors was small and the main visitors to the two plants were the same as those in their own separate populations. Therefore, although it was incomplete, there was a pollinators ethological isolation between the two species that contributed to the reproductive isolation between the two plants.

Reproductive isolation is essential for the process of speciation (Widmer et al., 2009) and is the basis of the biological species concept (BSC; Dobzhansky, 1940; Mayr, 1942). For *P. beesiana* and *P. bulleyana*, although there are different ideas regarding their classification, the present study suggests that there is reproductive isolation between the two species, which supports the hypothesis that *P. beesiana* and *P. bulleyana* are two distinct species. However, morphological similarity and interfertility suggest that they are incipient and closely related species pairs. Because there are no fossil records of *Primula*, we are unable to deduce the evolutionary history of these two species. It is possible that *P. bulleyana* and *P. beesiana* diverged from an unknown ancestor and then adapted different pollinators and occupied different places.

In the region of the Himalayas and western China, many *Primula* species are sympatric or partially sympatric in distribution; these plants usually differ in floral traits and remain distinct from each other. However, we are unable to draw a definitive conclusion based on the results of the present study for *P. beesiana* and *P. bulleyana* that ethological isolation is the most important mechanism for reproductive isolation in *Primula*. Further studies of reproductive isolation of many sympatric or partially sympatric closely related species pairs are required. The differences in the floral traits between closed species pairs of *Primula* in this area clearly suggest that the mechanisms of reproductive isolation between *P. beesiana* and *P. bulleyana* (ethological isolation) may be of general importance in maintaining species boundaries in the genus and has contributed to the species diversification of *Primula* in the area.

Natural hybridization is common in flowering plants, with one recent survey concluding that hybrids comprise 6–22% of all angiosperm species (Ellstrand et al., 1996). In *Primula*, most species can inter-cross in the same section, but it is unusual to find hybrids in the field. At the overlapping population site of *P. beesiana* and *P. bulleyana*, we found a few individuals with a medium color between the two species.

Natural hybridization possibly occurred in this population to some extent, but we did not find the medial type occupying a new habitat or site. Therefore, although gene exchanges between *P. beesiana* and *P. bulleyana* occur to some extent in the overlapping population, whether natural hybridization has contributed to species diversification in *Primula* remains unclear and requires further comparative studies on many species pairs and their hybrids to resolve.

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