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Asymmetrical disassortative pollination in a distylous primrose: the complementary roles of bumblebee nectar robbers and syrphid flies

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Heterostyly is a floral polymorphism characterized by reciprocal herkogamy maintained through high levels of mating between morphs, serviced by appropriate pollinators. We studied how differential efficiency and abundance of distinct pollinators affect plant female reproduction in self- and intra-morph incompatible distylous *Primula secundiflora*. Bumblebees and syrphid flies were found to be the most abundant floral visitors. Bumblebees frequently exhibited nectar-robbing behavior. Because the robbing holes were always situated between the high- and low-level organs on both morphs, nectar-robbing bumblebees only pollinated S-styled flowers. L-styled flowers set four times as many seeds as did S-styled flowers after being visited by pollen-collecting syrphid flies. The natural female fecundity and the magnitude of pollen limitation varied between the morphs within populations because of the mosaic distribution of nectar-robbing bumblebees and syrphid flies. L-styled flowers and S-styled flowers set the same number of seeds after supplemental hand pollination, indicating equivalent female reproductive potential. We suggest that bumblebee nectar robbers and syrphid flies play an important role in sustaining the floral dimorphism of heterostyly in *P. secundiflora* because of their complementary roles in the pollination system.

Heterostyly is a genetically controlled floral polymorphism characterized by reciprocal placement of stigmas and anthers between two (distyly) or three (tristyly) floral morphs of a species (reciprocal herkogamy)^{1–3}. It occurs in approximately 28 families of angiosperms, and distyly is much more common than trististyly^{2,4}. Individual plants of distylous species produce flowers with either long styles and short stamens (L-styled) or short styles and long stamens (S-styled), and most distylous species are self- and intra-morph incompatible^{2,5}. This reciprocal herkogamy is thought to function as a mechanism to increase the precision of inter-morph (disassortative) pollen transfer and to reduce lost mating opportunities associated with self-interference^{1–3,6}. Nevertheless, the efficiency of reciprocal pollination depends on the efficient services of pollinators, and it has been suggested that changes in pollination efficiency were responsible for the origin of heterostyly or for its breakdown^{7,8}.

When pollen transfer between floral morphs is highly asymmetrical, differential reproductive success between morphs can appear and has been emphasized as an important reason for the evolutionary breakdown of heterostyly. Asymmetrical disassortative mating increases male fitness for one morph and female fitness for the reciprocal morph and might lead to sexual specialization of morphs^{9–11}. In other cases, some heterostylos species present a self-incompatibility system that permits both intra- and inter-morph mating. Greater reproductive success of one morph due to significant intra-morph mating makes that morph more common, resulting in anisoplethic populations^{12–17}. The cause of pollen transfer asymmetries lies in the interaction between floral morphology and the entry and exit paths of pollinators^{2,18,19}. The pollen grains of the two morphs are usually carried on different body parts of legitimate long-tongued pollinators^{1,20}, but this does not guarantee that pollen transfer efficiency will be exactly the same between the two height levels^{21–23}. Shorted-tongued pollen collectors generally transfer more pollen grains between high-level organs^{8,14,24}. Zhu *et al.*²⁵ revealed that nectar robbers pollinate only S-styled flowers. Although most studies have revealed only one direction of asymmetry in pollen flow, it is possible that different kinds of asymmetries in pollen flow may occur simultaneously within populations



and/or among populations as a result of the differences in the body morphology and behavior of pollinators¹⁴. In this situation, it is interesting to study how the differential efficiency and abundance of distinct pollinators affect plant sexual reproduction and shape the evolution of heterostyly²⁶.

Since Darwin¹, *Primula* L. has been studied as a model for heterostyly, but pollination biology has been investigated thoroughly in only a few species. The Hengduan Mountains and the adjacent area are the distribution centre of this genus, with more than 75% of the total number of species (~425) being found in this region^{27,28}, though few studies have focused on them^{29,30}. *Primula secundiflora* is one of the most widely distributed primrose species in this region. Zhu *et al.*²⁵ found that bumblebees were the most abundant pollinators in the heart of the Hengduan Mountains, in Shangri-La, Yunnan, China. Bumblebees frequently acted as nectar robbers, either chewing a hole through the corolla tube or reusing an existing hole, and inserting their tongues to remove nectar produced by nectaries below the ovaries. The robbing holes were always situated between the high- and low-level organs for both floral morphs, and only S-styled flowers set seeds after legitimate pollinators were excluded. However, our preliminary experiments showed that the natural seed production of S-styled morphs was not uniformly greater than or equal to that of L-styled morphs in sampled populations. The fact that L-styled morphs set more seeds than S-styled morphs might be the result of higher level of intra-morph mating in L-styled morphs³¹. However, *P. secundiflora* is strongly self- and intra-morph incompatible³². Therefore, we hypothesized that there are other pollinators which cause asymmetrical disassortative pollination. In this work, we investigated pollinator assemblages, evaluated the pollination efficiency of pollinators, and compared female fecundity in six populations. We

focused primarily on the following questions: (1) Do floral visitor assemblages differ among populations? (2) Do the pollinators differ in their efficiency on both morphs? (3) How does this affect natural fruit and seed production?

Methods

Study species. *Primula secundiflora* is a distylous perennial herb that is widely distributed in the alpine regions of southwestern China^{28,33}. It produces leaves in a basal rosette and normally exhibits three to 43 flowers in a single umbel. The rosettes are sometimes divided but do not form extensive clones. Field experiments were conducted at Bigutianci (B), Napaihai (N), and Potatso National Park (PNP) in Shangri-La County, Yunnan Province, China. We studied three populations at B, two populations at N, and one population at PNP (Table S1 & Fig. 1). *Primula secundiflora* was the dominant species at the six sites, with thousands of individuals being present (Fig. S1).

Morph ratio and floral visitors. To assess whether the frequencies of the L-styled and S-styled morph differed from the expectation of 1 : 1, we recorded the morph of at least 400 flowering plants in each population. In 2011 and 2012, we conducted floral visitor observations in the six populations. We set four or five 2 × 2 m plots in each population. Each plot contained approximately 200 plants. Visits were recorded during a series of 15–30 min surveillance sessions under sunny conditions on different dates, which were evenly distributed through the floral season. All data were subsequently converted to the mean number of visits/plot/hour for analysis. We used two-way crossed analyses of similarities (ANOSIM) in the Primer 6 program to test for significant differences in the pollinator assemblages among populations and between years, with 10 000 permutations.

Pollination efficiency. Overall, the two most frequent floral visitors were the bumblebee *Bombus richardsi* and the syrphid fly *Rhingia binotata*. Because these two pollinators were frequently observed in population B2; thus, we conducted pollination efficiency experiments in this population. We enclosed a plot in a 1.5 × 2 × 2 m mosquito net supported by a plastic frame to prevent visitation. On different dates, we opened the netting to allow free-foraging insects to make single visits to the flowers. Because of the divergent behavior of legitimate *B. richardsi* and nectar-robbing *B.*

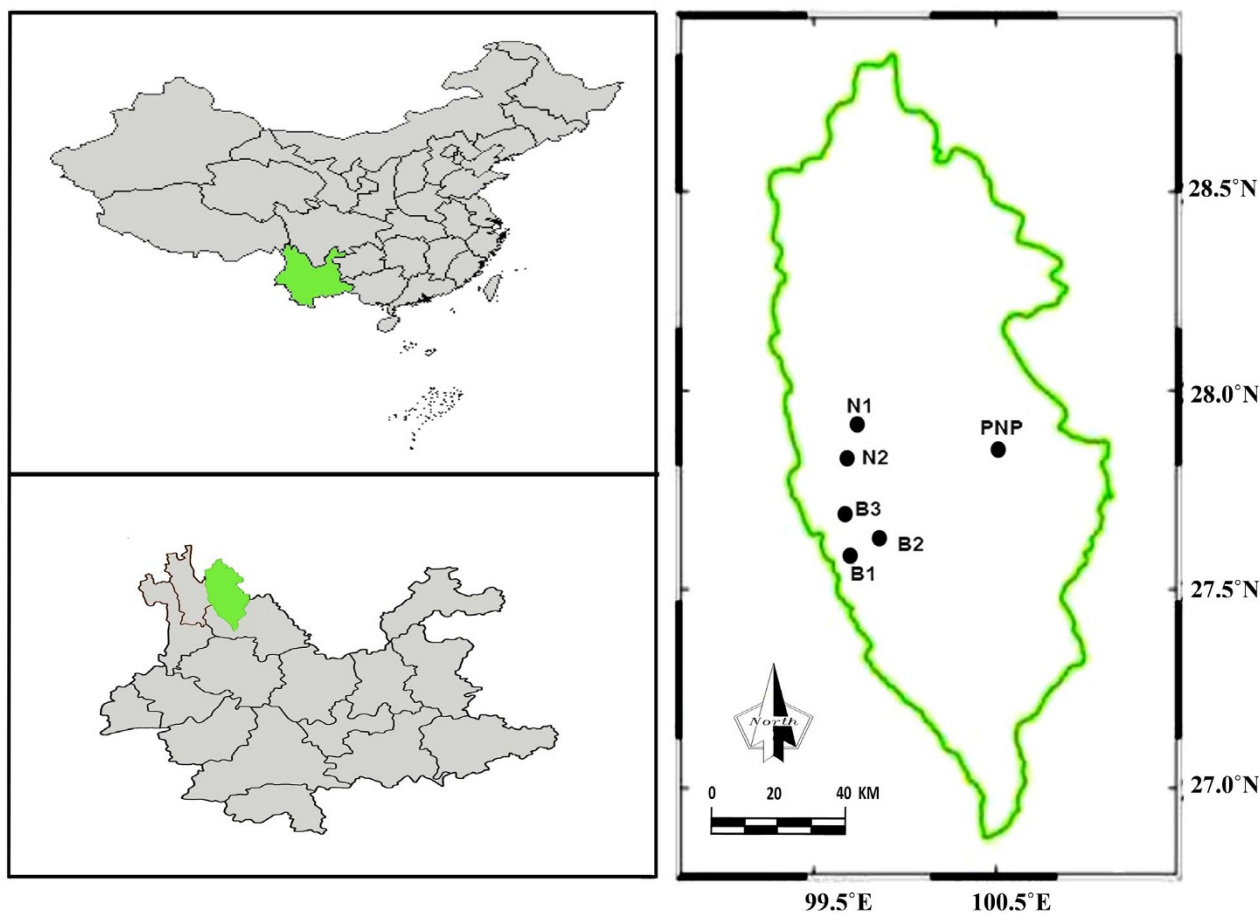


Figure 1 | Locations of the studied populations of *Primula secundiflora* in Shangri-La, Yunnan, China. The maps were generated using ArcGIS 10.0 software.



richardsi, we ascribed them to different pollinator types. Finally, we marked 42–60 flowers that were visited by each pollinator type for each morph. One month later, we measured the fruit and seed sets of the marked flowers. We measured the pollination efficiency of *B. securus* in population N2 using the same method. We attempted to study the pollination efficiency of the other floral visitors, but their low frequencies made this impossible.

Natural seed output. To determine whether the morphs differ in their seed output, we quantified natural fruit initiation, total seed production per plant, and number of seeds per fruit in populations B1, B3, and N1 in 2011 and in all six populations in 2012. We marked 30–41 plants with only one inflorescence for each morph. During flowering, we recorded the number of flowers produced by each plant. We collected all mature fruits prior to dehiscence. We counted the total number of seeds in each fruit. We performed MANOVA to clarify the effects of the morph, population and year on the flower number, fruit set, total seed production, and seeds per fruit. Because these components of seed output are likely to be intercorrelated, MANOVA was used to reduce the probability of type I error³⁴. A significant MANOVA was followed by univariate ANOVAs. The data on the number of seeds per fruit were normally distributed, whereas the other traits were transformed prior to statistical analysis. Flower number and total seed production were log transformed, and fruit set was arcsine square-root transformed.

Pollination treatments. To examine whether female reproductive potential differed between floral morphs and whether the seed production of *P. secundiflora* was pollen limited, we marked 35–56 plants of each morph in each population in 2012. Two flowers from each plant were pollinated with compatible pollen collected 10 m away from the focal plant. Another two flowers from each plant were selected as controls and left to be naturally pollinated. We directly brushed dehiscing anthers across receptive stigmas to pollinate L-styled flowers. For S-styled flowers, we punctured the corolla tube near the stigma and brushed dehiscing anthers across receptive stigmas through the hole using tweezers. Our pilot experiments showed that this treatment did not affect seed production (Mann–Whitney U-test, $P = 0.872$). To exclude the possible influence of limitation by resource allocation on seed production, we removed most of the untreated fruit when the experiments were set up. In most cases, we left ten flowers on each plant, including the four treated flowers. Ripe fruits were collected before dehiscence and brought back to the laboratory to determine the seed number. We used a logistical model and two-way ANOVA to examine the effects of the morph and population on fruit set and seeds per fruit in flowers subjected to supplemental pollination. For each plant, a pollen limitation index was calculated as $100 \times (PS - C)/PS^{35}$, where PS is the seed number of pollen-supplemented flowers, and C is the seed number of control flowers. The obtained values range from 0 to 100, with pollen limitation index = 0 indicating no pollen limitation. We used a two-way ANOVA with the population and morph as fixed factors to compare the difference in the magnitude of pollen limitation.

Results

Morph ratio and floral visitors. The morph ratio of L-styled to S-styled plants did not differ statistically from 1:1 in the six populations (G test: $G = 1.023$, d.f. = 5, $P = 0.961$). We carried out a total of 334 censuses corresponding to 134 h of observations, and recorded 2367 visits in the six populations over the two years. *Bombus richardsi* and *R. binotata* were the most abundant visitors (Fig. 2), accounting for 62.2% and 23.2% of total visits, respectively.

Bombus richardsi visited flowers to collect nectar in either a legitimate or nectar-robbing manner (Fig. S1). *Rhingia binotata* visited flowers to collect pollen grains and preferred to visit newly opened flowers, usually spending 2–5 min on a single flower. The other frequent visitors included *B. securus*, *B. festivus*, and a species of *Anthophora*. These bees visited flowers to obtain nectar at a rapid rate of approximately 2–3 s per flower. In addition, we observed two visits from a hawkmoth and two visits from a honey bee in population B2, four visits from butterflies in population N1, and three visits from *B. lucorum* and one visit from a small pollen-collecting solitary bee in population PNP. There was no significant difference in visitor abundance between the floral morphs (all floral visitors, G test: $G = 8.448$, d.f. = 5, $P = 0.585$). ANOSIM tests showed that the pollinator assemblages differed significantly among populations (Global R = 0.667, $P < 0.001$) but did not differ between years (Global R = 0.025, $P = 0.135$). All of the pair-wise comparisons of pollinator assemblages between populations were significant (R values ranged from 0.302 to 1, $P < 0.001$), except the comparison between populations B1 and B3 ($R = 0.004$, $P = 0.441$). *Rhingia binotata* was the most abundant pollinator in populations B1 and B3, whereas *B. richardsi* was the most abundant pollinator in populations N1, N2, and PNP. *Bombus richardsi* and *R. binotata* were both frequent in population B2 (Fig. 2). Some of the visits from *B. richardsi* were made by nectar-robbing individuals, with even more visits from nectar-robbing *B. richardsi* than from legitimate *B. richardsi* being recorded in population PNP (Wilcoxon signed ranks test, $z = 3.359$, $P < 0.001$).

Pollination efficiency. Only S-styled flowers set seeds after being visited by nectar-robbing *B. richardsi*. The L-styled morph exhibited a greater fruit set (chi-squared test, $\chi^2 = 31.73$, $P < 0.001$) and more seeds per fruit (t test, $t = 1.695$, d.f. = 60, $P = 0.047$) than the S-styled morph after a single visit by *R. binotata*. The L-styled and S-styled morphs showed a similar probability of setting fruit ($\chi^2 = 1.48$, $P = 0.224$ and $\chi^2 = 0.54$, $P = 0.513$, for *B. richardsi* and *B. securus*, respectively) and exhibited a similar seed set per fruit ($t = 0.296$, d.f. = 99, $P = 0.768$ and $t = 0.404$, d.f. = 56, $P = 0.688$, for *B. richardsi* and *B. securus*, respectively) after a single visit by legitimate bumblebees (Fig. 3).

Natural seed output. The three-way full factorial MANOVA indicated significant effects of the floral morph and population, but not of the year on the components of seed output (floral morph: Wilks's $\lambda = 0.90$, $P < 0.001$; population: Wilks's $\lambda = 0.26$, $P < 0.001$, year: Wilks's $\lambda = 0.99$, $P = 0.07$). We therefore proceeded with univariate analyses. Flower number did not differ significantly

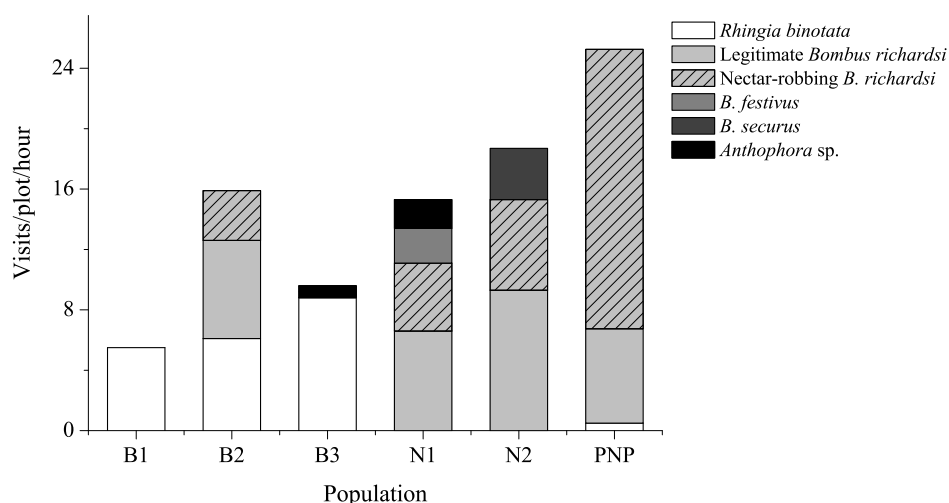


Figure 2 | Pollinator observations conducted in six *Primula secundiflora* populations.

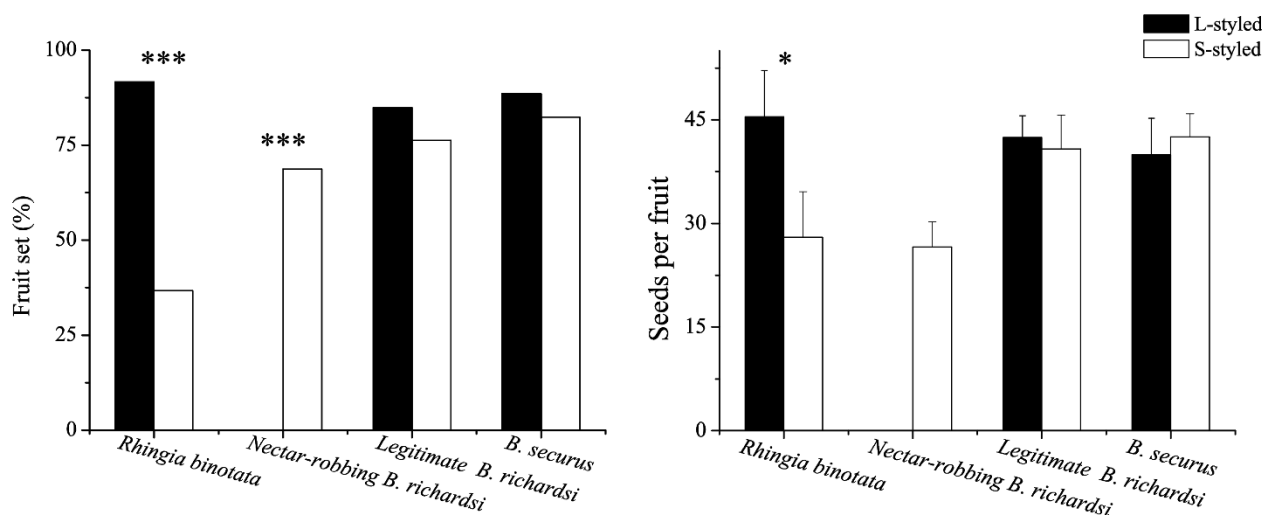


Figure 3 | Fruit set and seeds per fruit (mean + SE) recorded for L-styled and S-styled flowers after one visit by four pollinator types. Bars with asterisks indicate a significant difference between morphs (* $P < 0.05$, *** $P < 0.001$).

between the morphs or between the years, though it did differ significantly between populations. Fruit set, total seed number, and number of seeds per fruit were significantly different across populations and were significantly affected by the morph \times population interaction (Table 1). Fruit set, total seed production, and number of seeds per fruit for the L-styled flowers were greater than for the S-styled flowers in populations B1 and B3. This trend was reversed in populations N1, N2, and PNP. Fruit set, total seed production, and number of seeds per fruit were not significantly different between the morphs in population B2 (Fig. 4). These components of seed output did not vary between years, but the population \times year interaction significantly affected the fruit set and total seed number (Table 1) because the plants produced more seeds in 2011 than in 2012 in population B3, while they produced more seeds in 2012 than in 2011 in population B1 (Fig. 4).

Pollination treatments. After compatible pollen was added, most flowers set fruits (Fig. 5). Fruit set was not significantly different between the floral morphs ($\chi^2 = 0.841$, d. f. = 1, $P = 0.359$) and between populations ($\chi^2 = 7.267$, d. f. = 5, $P = 0.202$). The number of seeds per fruit also did not differ between the morphs ($F_{1,1114} = 2.51$, $P = 0.11$), though this parameter varied significantly between populations ($F_{5,1114} = 150.37$, $P < 0.001$). The magnitude of pollen limitation did not differ between morphs ($F_{1,570} = 0.84$, $P = 0.36$), but it did vary significantly between populations ($F_{5,570} = 39.65$, $P < 0.001$) and was significantly affected by the population \times morph interaction ($F_{5,570}$, $P = 0.001$). Pollen limitation in the L-styled morph was higher in N2 and lower in B1 and B3. No

significant difference between the L-styled morph and S-styled morph in terms of pollen limitation was found in B2, N1, and PNP (Fig. S2).

Discussion

Nectar-robbing *B. richardsi* bumblebees only pollinated S-styled flowers, whereas pollen-collecting *R. binotata* syrphid flies pollinated more L-styled flowers. Therefore, two kinds of asymmetrical disassortative pollination which could balance each other occur in *P. secundiflora*. Our data suggests that the nectar robbing bumblebees and the pollen-collecting syrphid flies are necessary for the proper functioning of the floral dimorphism because of their complementary roles in the pollination system. These species certainly play an important current role in sustaining the floral dimorphism of distyly in *P. secundiflora*.

Bumblebees are well-known pollinators of primroses because of their high visitation speed and long proboscis, which easily reaches the nectar at the base of a long corolla tube. Washitani *et al.*²⁰ demonstrated that bumblebees facilitate inter-morph crossing in *P. sieboldii*, with high fruit and seed sets being recorded for both floral morphs. L-styled flowers and S-styled flowers set the same number of seeds after being visited by legitimate bumblebees in this study. It is very possible that legitimate bumblebees transfer pollen grains efficiently in both directions, and the flowers of primroses are well adapted to bumblebee pollination. However, nectar-robbing bumblebees only pollinated S-styled flowers in *P. secundiflora*. Darwin³⁶ also noted that bumblebees visiting the flowers of *P. veris* exhibited

Table 1 | Testing the effects of the Population, Morph (L-styled vs. S-styled) and Year on the flower number, fruit set, seeds per fruit, and total seed production per plant in a field experiment conducted in Shangri-La, China, in 2011 and 2012. Asterisks denote significant differences (** $P < 0.01$, *** $P < 0.001$)

Source	Flower number			Fruit set		Total seed production		Seeds per fruit	
	df	MS	F	MS	F	MS	F	MS	F
Population	5	1.16	37.50***	5.47	96.36***	17.73	140.28***	71311.74	151.65***
Morph	1	0.02	0.58	0.15	2.58	0.28	2.20	4826.35	10.26**
Year	1	0.07	2.19	0.11	1.88	0.45	3.57	71.9	0.15
Population \times Morph	5	0.02	0.69	0.85	14.94***	1.70	13.44***	6570.57	13.97***
Population \times Year	2	0.06	1.81	1.74	30.58***	2.21	17.46***	221.85	0.47
Morph \times Year	1	0.03	1.01	0.05	0.81	0.01	0.08	553.36	1.18
Population \times Morph \times Year	2	0.03	1.01	0.03	0.54	0.28	2.20	559.70	1.19
Error	624	0.03		0.06		0.13		470.23	

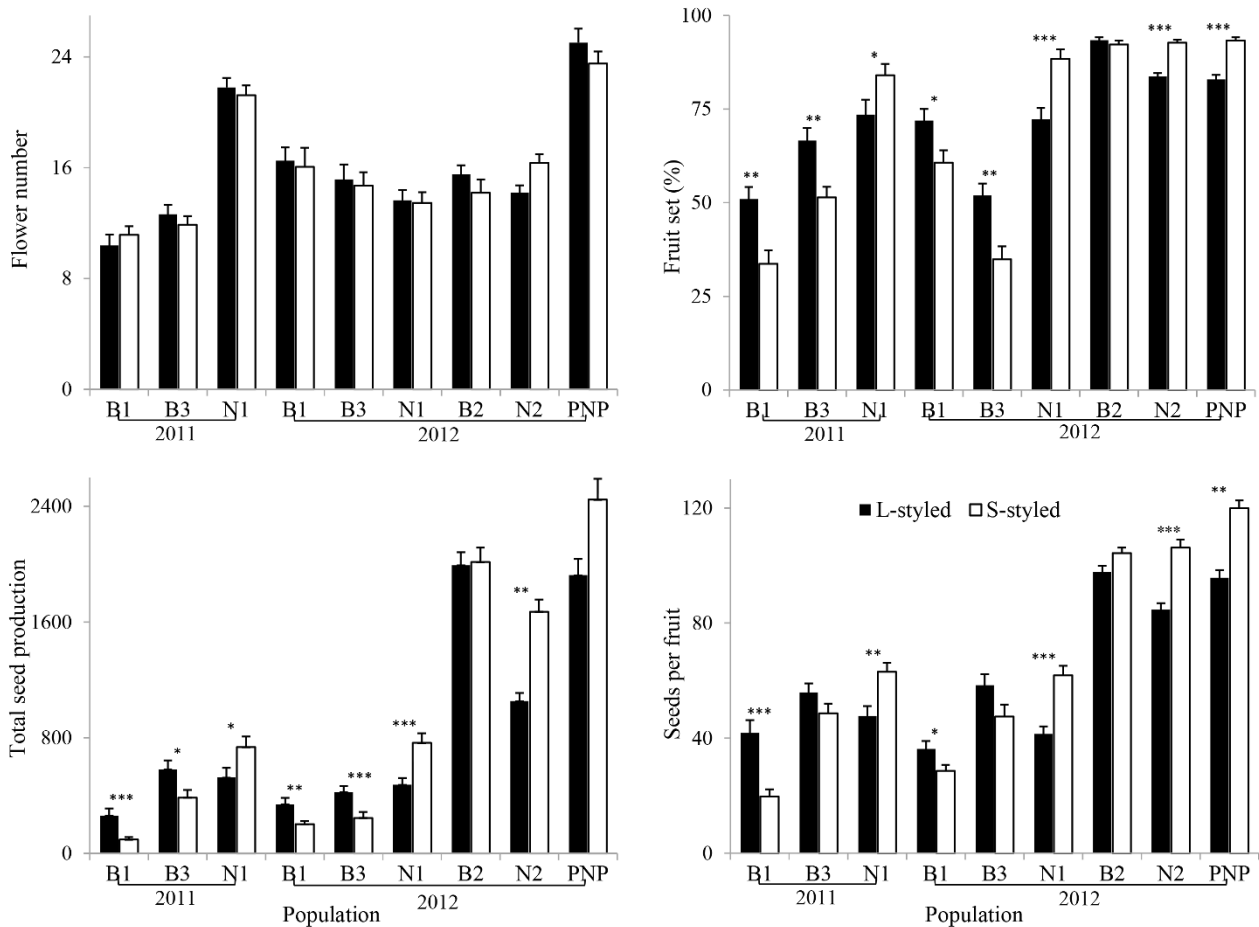


Figure 4 | Flower number per plant, fruit set, total seed output per plant, and seed number per fruit on *Primula secundiflora* (mean + SE) in six populations in 2011 and 2012, in Shangri-La, Yunnan, China, examining the effects of the morph and population in different pollination environments ($n = 30\text{--}41$). Bars with asterisks indicate a significant difference between morphs (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

robbing behavior. In the field, we observed that *P. sikkimensis* and *P. amethystine* suffer some degree of nectar robbing as well. It is likely that nectar robbing in other primrose species also transfers pollen grains only between low-level organs. Syrphid flies usually feed on both nectar and pollen³⁷. The length of the *R. binotata* proboscis was found to be 5.9 ± 0.1 mm ($n = 8$), which is long enough to reach the

low-level organs (depths: 4 ~ 5 mm), but not the nectar (corolla tube length: 8 ~ 9 mm)³⁸. Hence, the proboscises of these flies preclude them from reaching the nectar but allow them to collect pollen grains from both floral morphs. When the flies access the anthers, they have to pass through the stigmas of L-styled flowers, whereas they might not contact the hidden stigmas of S-styled flowers, resulting in a

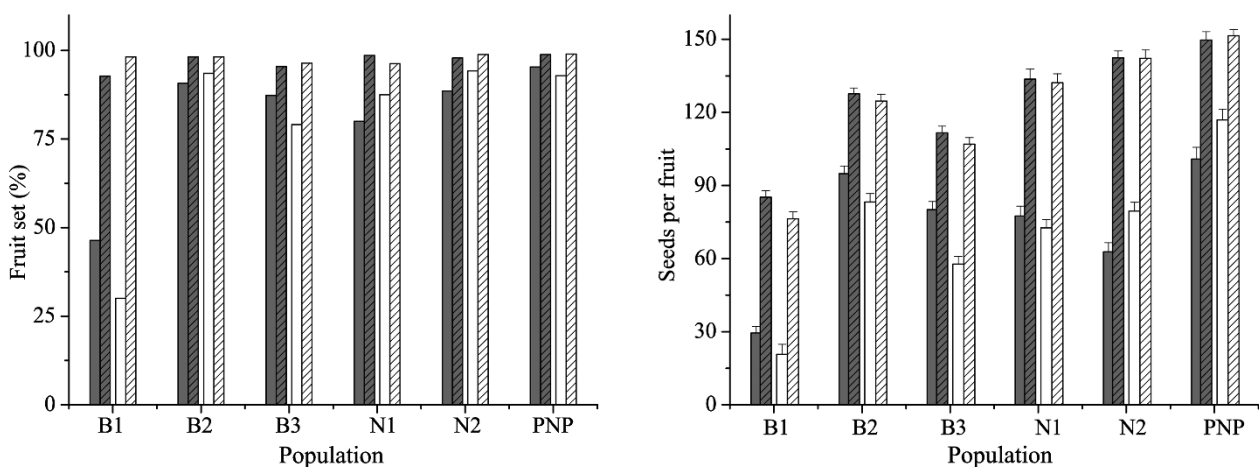


Figure 5 | Fruit set and seeds per fruit recorded for L-styled (black gray) and S-styled (white) flowers subjected to natural pollination (solid bars) or supplemental pollination (hatched bars) in the six populations. Data are means + SEs.



greater amount of pollen transfer between high-level organs. We did not test the pollination efficiencies of other visitors, and some of these pollinators might also cause asymmetric pollen flow.

The abundance of *R. binotata* syrphid flies and *B. richardsi* bumblebees could largely explain the difference in natural female fecundity between floral morphs. When *R. binotata* was dominant in populations B1 and B3, fruit set, total seed production, and seeds per fruit of the L-styled flowers exceeded those of the S-styled flowers. With some individuals acting as nectar robbers, *B. richardsi* was the most frequent pollinator in populations N1, N2, and PNP, while this trend was reversed in S-styled flowers, where greater fruit set, total seed production, and seeds per fruit were observed. It is interesting that female fecundity was not different between the morphs in population B2, where *R. binotata* and *B. richardsi* were both frequent visitors (Figs. 2 & 4). The difference in pollen limitation between the morphs also partly reflected how the pollinator assemblage affected natural seed production. S-styled plants were more pollen limited in the two *R. binotata*-dominated populations, whereas the only population where L-styled plants were more pollen limited was N2 in which *B. richardsi* was the most abundant pollinator (Figs. S2). Fang and Huang³⁹ reported that *R. binotata* visited the flowers of eight species near population N2, and we observed that this species visited the flowers of *Cynoglossum amabile* and *Iris bulleyana* frequently in population PNP, whereas it seldom visited flowers of *P. secundiflora* at these two sites. These findings show that other floral species might affect the behavior of *R. binotata* and determine whether it visits the flowers of *P. secundiflora*⁴⁰. Nevertheless, it is worth identifying the factors that cause the mosaic distribution of pollinators and ultimately result in the differences in sexual production between morphs.

Although it is common for the pollen transfer pattern to be unequal between the two directions in heterostylous species⁴¹, gender specialization is not frequent². In the present study, L-styled flowers and S-styled flowers set the same number of seeds after pollen was added, indicating equivalent female reproductive potential. If no self- or intra-morph fertilization occurs, the average gender of the morphs can be estimated from their seed production alone; in this situation, the average femaleness of one morph is exactly equal to the average maleness of the other morph⁹. The equivalence of female reproductive potential between morphs might imply that gender specialization does not occur in *P. secundiflora*. We suggest that the co-existence of L-biased asymmetrical disassortative pollination and S-biased asymmetrical disassortative pollination within and/or among populations contributes to the persistence of the floral dimorphism. First, it is likely that the coexistence of the two kinds of asymmetrical disassortative pollination would enable male and female functions to be equally realized for both morphs within populations, as indicated in population B2 (Fig. 3); thus, selective pressure for gender specialization does not exist. Second, the gene flow between populations that exhibit different directions of asymmetrical mating tends to oppose the effects of local selection and thus counteract gender specialization⁴², and the mosaic distribution of pollinators may reinforce this process. We did not find differences in the pollinator assemblages recorded within populations between the two study years. However, it is possible that over long periods of time the ratio between the natural female fecundity of the L-styled flowers and that of the S-styled flowers varied from year to year due to changes in the relative abundance of pollinators. Gonzalez et al.⁴³ reported inconsistent female fecundity across five years between floral morphs of *Palicourea padifolia* and speculated that this pattern was partly due to the variation of the relative frequency of hummingbird and pollen-collecting insects. In this system, it is known that hummingbird visitation promotes pollen flow from long-styled flowers to short-styled flowers²³; however, these authors did not monitor the pollinator assemblage or evaluate the pollination efficiency of pollen-collecting insects, which is believed to result in L-biased asymmetric disassortative

pollination. Taking these findings together, if L-biased and S-biased asymmetrical disassortative pollination occur simultaneously in distylous plants as a result of differential efficiency of distinct pollinators, gender specialization might hardly occur, owing to gene flow between populations with different direction of asymmetrical disassortative mating and fluctuating selection over long period of time, in spite of the fact that mating opportunities might differ between the two height levels within populations.

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Author contributions

X.F.Z. and Q.J.L. designed the research and wrote the manuscript; X.F.Z., X.F.J., L.L. and Z.Q.Z. performed experiments; X.F.Z. analyzed data and prepared the figures and tables.

Additional information

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