

ARTICLE



Inheritance of distyly and homostyly in self-incompatible *Primula forbesii*

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The evolutionary transition from self-incompatible distyly to self-compatible homostyly frequently occurs in heterostylous taxa. Although the inheritance of distyly and homostyly has been deeply studied, our understanding on modifications of the classical simple Mendelian model is still lacking. *Primula forbesii*, a biennial herb native to southwest China, is a typical distylous species, but after about 20 years of cultivation with open pollination, self-compatible homostyly appeared, providing ideal material for the study of the inheritance of distyly and homostyly. In this study, exogenous homobrassinolide was used to break the heteromorphic incompatibility of *P. forbesii*. Furthermore, we performed artificial pollination and open-pollination experiments to observe the distribution of floral morphs in progeny produced by different crosses. The viability of seeds from self-pollination was always the lowest among all crosses, and the homozygous S-morph plants (S/S) occurred in artificial pollination experiments but may experience viability selection. The distyly of *P. forbesii* is governed by a single S-locus, with S-morph dominant hemizygotes (S/—) and L-morph recessive homozygotes (—/—). Homostylyous plants have a genotype similar to L-morph plants, and homostyly may be caused by one or more unlinked modifier genes outside the S-locus. Open pollinations confirm that autonomous self-pollination occurs frequently in L-morphs and homostylyous plants. This study deepens the understanding of the inheritance of distyly and details a case of homostyly that likely originated from one or more modifier genes.

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INTRODUCTION

Transitions from obligate outcrossing to autonomous self-fertilization frequently occur in angiosperm species (Igic et al. 2006; Shao et al. 2019). However, in environments lacking pollinators and/or pollen donors, adaptive transitions toward selfing are favored and can be fixed in populations because of its reproductive advantage (Darwin 1876; Stebbins 1974; Busch and Delph 2012; Yuan et al. 2017). Changes in mating strategy can lead to reproductive isolation and thus are related to speciation at the population level (Wright et al. 2013). In plants, transitions from outcrossing to selfing are commonly accompanied by heritable directional shifts in floral morphology (e.g. reduced herkogamy and flower diameter) (Morgan and Barrett 1989; Sicard and Lenhard 2011; de Vos et al. 2014; Wu et al. 2017) and physiological traits (e.g. loss of self-incompatibility) (Mable et al. 2005; Igic et al. 2008; Zhong et al. 2019; Liu et al. 2022). Since Darwin's seminal work, determining the inheritance of traits that structure the variation and evolution of mating systems has been a focus of evolutionary biologists, ecologists and geneticists (Darwin 1876; Shore and Barrett 1985; Barrett et al. 2014; Igic and Busch 2013). After more than a century of study, it has been revealed that the inheritance basis of variation in mating systems includes both some key genes with large effects and some quantitative genes with small effects (Fishman et al. 2002; Matsui et al. 2004; Slotte et al. 2012; Yuan et al. 2019). In the present study, based on crosses of heterostylous plants and novel homostylyous plants, which present a paradigmatic model system for studying

transitions from outcrossing to selfing, the inheritance of traits related to mating system variation have been explored, providing an empirical basis for further genetic and ecological research of mating system evolution.

Heterostyly is a heritable floral polymorphism that includes two (distyly) or three (tristyly) floral morphs characterized by reciprocal positioning of stigmas and anthers (Darwin 1877; Barrett 1992; Lloyd and Webb 1992). Heterostyly has independently originated in at least 28 angiosperm families and 199 genera, particularly in tubular-flowered species with animal-mediated pollination (Barrett and Shore 2008). The major expression of heterostyly is distyly, in which the flowers of one morph have a high stigma and low anthers (long style morph, L-morph, L), while the other morph expresses the opposite pattern (short style morph, S-morph, S) (Darwin 1877; Barrett 2019). The vast majority of distylous species possess heteromorphic incompatibility systems, in which only inter-morph pollination is compatible, while both intra-morph pollination and self-pollination are incompatible (Barrett and Shore 2008; Brys and Jacquemyn 2015). Distyly has long been regarded as an ideal mechanism to promote outcrossing and improve pollen dispersal efficiency since Darwin's seminal work (Darwin 1877; Yuan et al. 2017). However, the discovery of numerous self-compatible homostylyous plants (H-morph, H) with stigmas and anthers at the same position in distylous populations suggests a new potential evolutionary response to this floral polymorphism (Darwin 1877; Zhou et al. 2017; Barrett 2019; Zhong et al. 2019). Shifts to homostyly frequently occur in alpine

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environments, in peripheral populations and during colonization, when pollinators and/or mates are not reliably available (Brys and Jacquemyn 2015; Yuan et al. 2017; Shao et al. 2019; Zhang et al. 2021; Liu et al. 2022). Therefore, under conditions averse to outcrossing, distylous populations are forced by selection pressure to abandon obligate outcrossing and transition to self-pollination (Busch and Delph 2012). Although such transitions to selfing have ensured the survival and reproduction of species, they are frequently accompanied by inbreeding depression. Self-fertilization leads to higher homozygosity and the expression of deleterious recessive alleles, which eventually leads to reduced seed mass, germination rates, seedling vigor and reproductive capacity (Luijten et al. 2002; Charlesworth and Willis 2009; Barmentlo et al. 2018).

In the past century, many investigations of the inheritance of heterostyly and homostyly have focused on *Primula* (Bateson and Gregory 1905; Kappel et al. 2017). The genus *Primula* is comprised of ~430 species, with the vast majority (95%) being distylous and 45 being florally monomorphic (Richards 2003; Mast et al. 2006). Phylogenetic analysis of *Primula* clearly shows a single independent origin of distyly and multiple transitions to homostyly (Mast et al. 2006; de Vos et al. 2014; Zhong et al. 2019). The early genetic studies in *Primula* were mainly based on the findings of Bateson and Gregory (1905), who established that distyly was controlled by a single *S*-locus following Mendelian inheritance, with *S*-morph dominant heterozygotes (*S/s*) and *L*-morph recessive homozygotes (*s/s*). Then, based on numerous hybridization results of *L*-morph, *S*-morph and homostylous plants, Ernst (1955) constructed the *S*-locus supergene model comprised of at least three tightly linked genes, controlling style elongation, anthers position and pollen morphology, respectively. Therefore, researchers presumed that the homostyly frequently occurring in distylous population may have originated via homologous recombination (Pamela and Dowrick 1956; Lewis and Jones 1992; Barrett and Shore 2008). However, recent studies have demonstrated that the *S*-locus is

actually a hemizygous region only existing in the *S*-morph (*S/-*) and absent in the *L*-morph (*-/-*) (Li et al. 2016; Kappel et al. 2017; Shore et al. 2019; Mora-Carrera et al. 2023). Further studies showed that the *S*-locus of *Primula* contains at least five genes, with *CYP* controlling style length and female self-incompatibility and *GLO* controlling anther height (Huu et al. 2016; Huu et al. 2020). Based on the hemizygous genetic model, homostyly in this system cannot have originated via homologous recombination within the *S*-locus, because there are no homologous sequences on the *s* haplotype with which it could pair and crossover (Barrett 2019). Consistent with Ernst (1955), the observed homostyly probably arose from mutations of *S*-locus coding sequence, promoter region variation and/or gene structure rearrangements (Huu et al. 2016; Huu et al. 2020; Mora-Carrera et al. 2023). Through a less common pathway, homostyly may also arise from unlinked modifier genes outside the *S*-locus (Richards 1997; Sicard et al. 2011; Arunkumar et al. 2017; Barrett 2019). Under this evolutionary pathway, homostylous plants would exhibit considerable variation in the stigma and anther positions, which is more commonly reported in self-compatible species, rather than ones with strong self-incompatibility (Matsui et al. 2004; Brys and Jacquemyn 2015; Zhang et al. 2021).

Owing to the existence of heteromorphic incompatibility, the vast majority of previous studies on the inheritance of distyly and homostyly have been conducted on a few species that exhibit self-compatibility (Ernst 1955; Pamela and Dowrick 1956; Yuan et al. 2019). Some of the most recent research indicates style length in distyly is mainly developmentally determined by brassinosteroids, with the *L*-morph and *S*-morph containing high and low concentrations of brassinosteroids, respectively (Huu et al. 2016). Furthermore, brassinosteroids also play an important role in female self-incompatibility (Huu et al. 2022). The treatment of *S*-morph flower buds with exogenous brassinosteroids can induce 'long homostyly,' characterized by long styles and high anthers, rendering such flowers completely self-compatible (Huu et al. 2022). In the

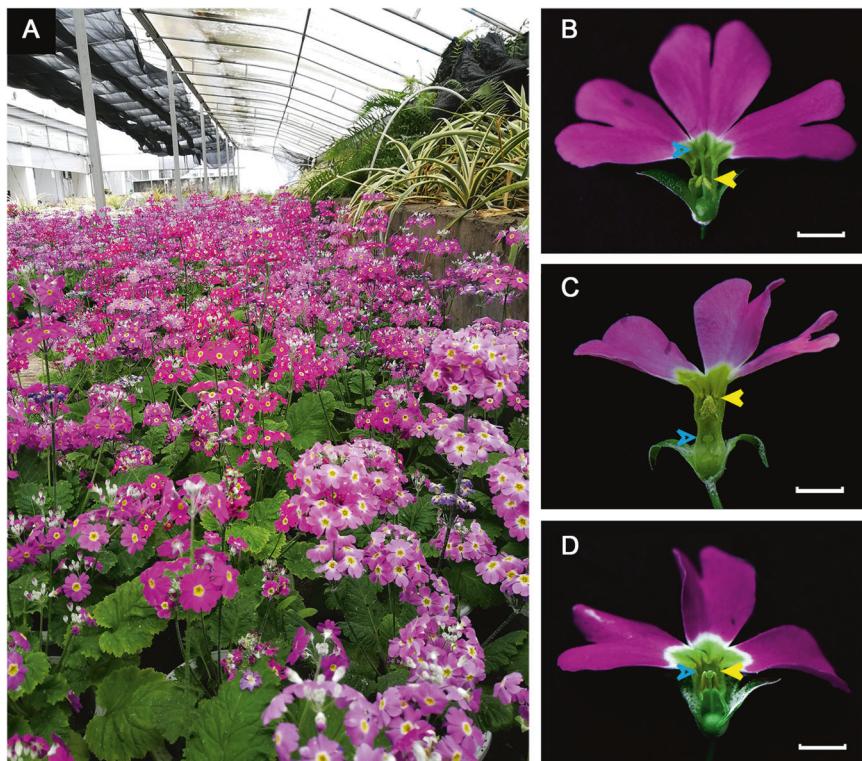


Fig. 1 Flowering plants and floral morphs of *Primula forbesii*. **A** Flowering plants. **B–D** Dissected L-morph, S-morph and H-morph flowers, showing differences in style length (blue arrowheads) and anther position (yellow arrowheads) (scale bar = 3 mm).

present study, through exogenous brassinosteroid treatment, we provide more comprehensive cross evidence elucidating the inheritance of distyly with strong heteromorphic self-incompatibility.

Primula forbesii Franch. (Primulaceae), a typically distylosus species with L-morph that is partially self-compatible and S-morph that is completely self-incompatible (Huu et al. 2022; Liu et al. 2022), is a popular horticultural flower because of its pleasant fragrance and abundant purple flowers (Fig. 1A–C). However, preliminary observations revealed that after about 20 years of cultivation with open pollination in a greenhouse, a distylosus population of *P. forbesii* eventually came to exhibit a broader range of floral morphs, producing homostylosus plants with stigma and anthers in the same position (Fig. 1D). These variants provide an ideal material for the study of the inheritance of distyly and homostyly in *P. forbesii*. In order to harvest seeds produced by intra-morph pollination and self-pollination of S-morph plants, exogenous homobrassinolide (homo-BL) was injected into flower buds to render them self-compatible (Huu et al. 2022). In this study, we mainly focused on answering the following three questions. (1) What are the inheritance laws of distyly and homostyly? We aimed to determine whether homostyly had arisen by mutation of key genes at the S-locus or by one or more unlinked modifier genes outside the S-locus. (2) For partially absent heteromorphic incompatibility, does the disassortative mating system mediated by pollinators break down in *P. forbesii*? We predicted that autonomous self-fertilization would occur frequently in both L-morph and H-morph plants, with the latter being more obvious because of the proximity of the stigma to the anthers. (3) Is inbreeding depression strong in *P. forbesii* and at what stage is it manifested? Because self-fertilization may lead to the expression of deleterious recessive genes, we predict that the seeds resulting from self-pollination may have lower viability. In addition, owing to the intrinsic hemizygosity of distyly in this system, the S-locus may be linked with recessive lethal genes, such that homozygous S-morph plants (SS) are likely to die or experience severe viability selection. This prediction originates with the work of Richards (1997) and has found only limited support elsewhere (Yuan et al. 2019). In the present work, we tested the hypothesis and found support for this prediction by performing intra-morph pollination and self-pollination of S-morph plants and observing the floral distribution of progeny.

MATERIAL AND METHODS

Primula forbesii is a subalpine biennial herb native to Yunnan, China, belonging to the section *Monocarpicae* of *Primula* (Chen and Hu 1990). In spring 2000, we collected flowering plants of *P. forbesii* from two wild distylosus populations at Heilongtan (102°45'14"E, 25°8'38"N) and Xishan (102°37'34"E, 24°58'24"N) in Yunnan, China. All plants were then cultivated in the greenhouse at Sichuan Agricultural University, Chengdu Campus (103°51'44"E, 30°42'17"N). During greenhouse cultivation, no directional breeding was conducted, and the open-pollinated seeds were randomly harvested and sown every year. In spring 2018, we observed homostylosus variants for the first time in the previously exclusively distylosus population of *P. forbesii*.

For this study, seeds of *P. forbesii* were randomly harvested in April 2020 after natural open pollination and were sown into seedling trays in a greenhouse in July. One month later, more than 3000 of the resulting seedlings were transplanted into plastic pots (10 cm × 15 cm) filled with peat soil. Plants were watered every 3 days, and the light, temperature and humidity of the greenhouse were not artificially manipulated. At flowering, we randomly selected 300 plants to investigate the floral morph ratio of the population. The χ^2 -test implemented in SPSS 23.0 (IBM Corp., Armonk, NY, USA) was used to determine whether the L-morph and S-morph frequencies had deviated from the expected ratio of 1:1.

Brassinosteroid treatment

For the brassinosteroid treatment experiment, we selected at least 150 flowers of 30 L-morphs and 150 flowers of 30 S-morphs of *P. forbesii*. According to the methods of Huu et al. (2016), three experiment groups were established: (1) 10 μ M homobrassinolide (homo-BL) (dissolved in dimethyl

sulfoxide), (2) mock solution containing 0.1% dimethyl sulfoxide, consistent with the concentration of dimethyl sulfoxide in the 10 μ M homo-BL solution, and (3) untreated. We injected flower buds (with petals of flower buds measuring 3 mm in length) every other day with the above solutions until they reached full flowering. After flowering, 50 flowers of each treatment were slit longitudinally and digitally photographed, and then, from those digital images, ImageJ was used to measure the style length, from the top of the ovary to the stigma, and the height of the anthers, from the top of the ovary to the anthers. Student's *t* test was used to determine the significance of differences in the height of the stigma and anthers between flowers in the 10 μ M homo-BL treatment and control groups in SPSS 23.0.

Controlled pollinations

To investigate the effects of exogenous brassinosteroids on heteromorphic incompatibility in *P. forbesii*, L-morph and S-morph flower buds were treated according to the above methods. Two experiment groups were established: 10 μ M homo-BL application and mock application. After treatment, six artificial pollinations were conducted: inter-morph pollination (i.e., L × S and S × L) and both intra-morph pollination and self-pollination of L-morph and S-morph plants. For each pollination type, we collected approximately 20 plants and more than 150 flowers, and all flowers used were in their second or third day of blossoming. To exclude pollinators, flowers were bagged before blossoming and after artificial pollination, and for crossing treatments, all flowers were emasculated and bagged prior to blossoming. For each cross-pollination, pollen was collected from more than three flowering plants. All pollinations were performed in February 2021, and the fruits and seeds were gathered in April–May, at which time fruit set and seed production per fruit were recorded. Student's *t* tests, as implemented in SPSS 23.0, were used to determine the effectiveness of exogenous homo-BL on breaking self-incompatibility of *P. forbesii*. Furthermore, to investigate the inheritance of distyly in *P. forbesii*, seeds of different artificial pollinations of L-morphs and S-morphs were gathered and stored at 4 °C after being fully dried in air.

To determine the origination and inheritance of naturally arising homostyly, different hand pollinations of H-morphs were conducted using the above methods, including self-pollination, cross-pollination with other H-morph plants, and crossing with L-morph (i.e., H × L and L × H) and S-morph plants (i.e., H × S and S × H) (Yuan et al. 2019). Finally, the resulting seeds were gathered and stored as described above.

Thousand-seed weight and germination rate

To assay inbreeding depression in *P. forbesii*, the weight of one thousand seeds and rate of seed germination were measured. To measure the thousand-seed weight of each cross, 500 seeds from each treatment were randomly selected and weighed on an electronic balance, with three replicates of each treatment. To investigate the germination rate (Jiang et al. 2018), 100 healthy and plump seeds of each pollination type were selected. After rinsing them with distilled water three times, seeds were neatly placed in a Petri dish with wet filter paper. The culture dish was then placed in a cabinet, maintained at 20 ± 2 °C, 60% humidity and an illumination level of 3500 lx for 12 h. During the experiment, water was replenished regularly to keep the filter paper wet continuously. Each germination was recorded only if the radicle length exceeded the diameter of the seed. After 1 week, the germination test was ended once no seeds had germinated for 5 consecutive days. There were three replicates of each treatment. We used one-way analysis of variance (ANOVA) to identify significant differences in thousand-seed weight and germination rate among the different pollination types.

Inheritance of distyly and homostyly

The progeny derived from artificial outcrossing and self-pollination were used to determine the inheritance laws of distyly and homostyly in *P. forbesii*. In the greenhouse, the seeds of each pollination type were germinated on soil-filled seed trays in July 2021. After a month, 100 seedlings of each pollination type were transplanted into plastic pots (10 cm × 15 cm) filled with peat soil, and the plants were maintained using the above-described methods. At flowering, the floral morph ratio of F_1 progeny of each pollination type was recorded. We used χ^2 -tests, as implemented in SPSS 23.0, to determine whether the ratio of L-morph to S-morph progeny had deviated from the expected ratios of 1:1, 1:2 or 1:3. Furthermore, more than 20 flowers were randomly selected from progeny of each pollination. Flowers were slit longitudinally and digitally photographed, and the height of the stigmata and anthers were measured from the top of the ovary using ImageJ.

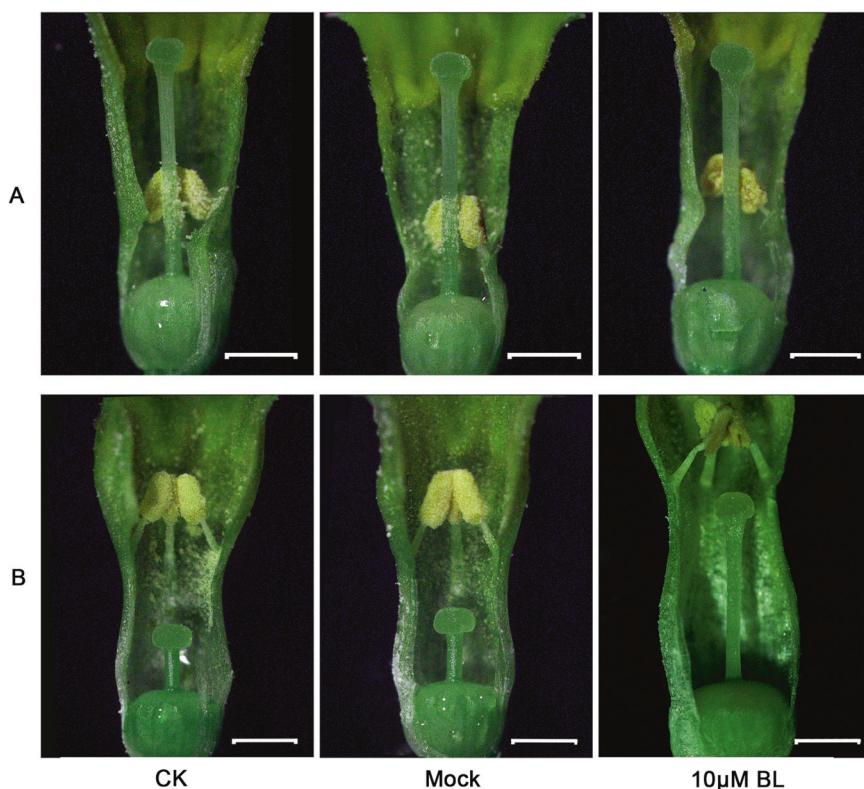


Fig. 2 Floral morphology of *Primula forbesii* under the indicated treatments. **A** L-morph flowers. **B** S-morph flowers.

Investigation of progeny from open pollination

To assess the mating style of different floral morph of *P. forbesii* under open pollination, 10 L-morphs, 10 S-morphs and 10 H-morphs were selected, and all of them were then naturally pollinated by insects in the greenhouse from February to May 2021. To make the conditions as realistic as possible, the 30 selected plants were not isolated from other plants. The seeds of each floral morph after open-pollination were harvested and stored as described above. We grew these seeds to mature flowering plants using the above methods and recorded the floral morph ratio of progeny of each maternal seed family. We used the χ^2 -test implemented in SPSS 23.0 to determine whether the frequencies of L-morph and S-morph plants had deviated from the expected ratio of 1:1.

RESULTS

Effects of exogenous brassinosteroid on floral morphology

Treatment with exogenous homo-BL significantly increased the height of the stigma and anthers of *P. forbesii* (Fig. 2 and Fig. 3). Compared with plants in the control group (3.32 ± 0.03 mm) and the mock treatment group (3.26 ± 0.05 mm), 10 μ M homo-BL treatments (4.09 ± 0.07 mm) significantly increased the style length of the S-morph (10 μ M homo-BL versus CK, $t = -9.80$, d.f. = 98, $P < 0.001$; 10 μ M homo-BL versus mock, $t = -9.98$, d.f. = 98, $P < 0.001$), and some individuals developed 'long homostyly' (Fig. 2B). However, the style length of L-morph plants did not show a similar response, suggesting that exogenous homo-BL especially promotes the growth of S-morph plants. Under exogenous homo-BL treatment, there were different degrees of increase in anther height in both L-morph and S-morph flowers.

Effects of exogenous homobrassinolide on heteromorphic self-incompatibility

The L-morph was partially self-compatible, with about 60% fruit set and 100 seeds per fruit under intra-morph pollination and self-pollination, and exogenous homo-BL treatments had little effect on the self-incompatibility of L-morphs (Fig. 4A, B). On the

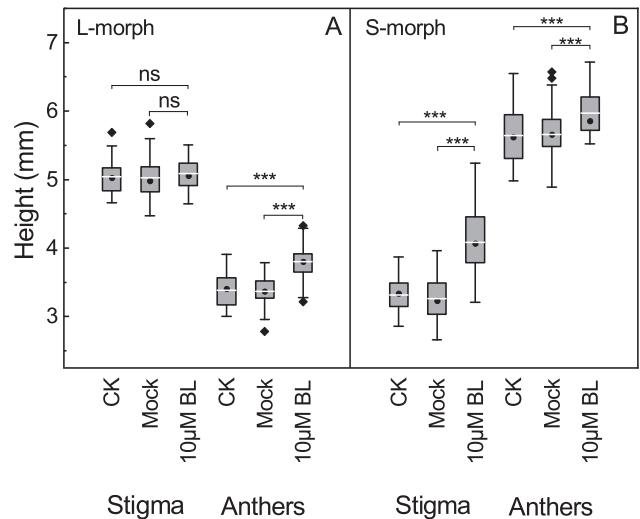


Fig. 3 Stigma and anther heights of *Primula forbesii* under the indicated treatments. **A** L-morph flowers. **B** S-morph flowers. Differences were not significant (ns), significant ($^*P < 0.05$) or very significant ($^{**}P < 0.01$; $^{***}P < 0.001$) based on Student's *t* test. Box edges indicate the lower and upper quartiles, respectively, the white middle line represents the median and the black dot and squares represent the mean value and abnormal values, respectively. The vertical bars indicate upper and lower limits of the data or extend to 1.5 times beyond the upper or lower quartile.

contrary, S-morph plants were completely self-incompatible, and 10 μ M homo-BL treatments induced a significant break down of the self-incompatibility system (Fig. 4C, D). For inter-morph pollinations, the homo-BL treatments significantly decreased the fruit set (0.77 ± 0.03) and seed production (172.80 ± 9.30) compared with plants subjected to mock treatments (fruit set

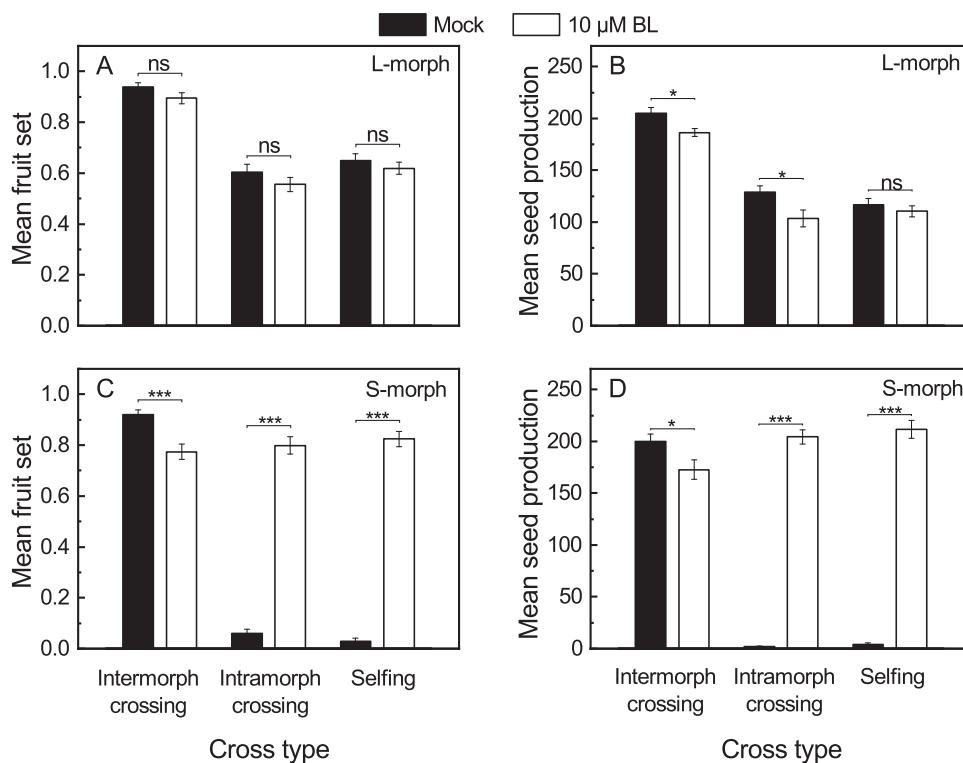


Fig. 4 Fruit set and seed production of the different floral morphs of *Primula forbesii* after inter-morph pollination, intra-morph pollination or self-pollination under indicated treatments. **A-B** L-morph plants. **C-D** S-morph plants. Differences were not significant (ns), significant ($*P < 0.05$) or very significant ($**P < 0.01$; $***P < 0.001$) based on Student's *t* test.

0.92 ± 0.19 , $t = 4.07$, $d.f. = 38$, $P < 0.001$; seed production 199.80 ± 7.28 , $t = 2.29$, $d.f. = 18$, $P = 0.04$). In contrast, unlike the mock treatment pollinations, in which almost no seeds were produced under intra-morph pollination and self-pollination, the S-morph plants treated with $10 \mu\text{M}$ homo-BL had become fully self-compatible, with high fruit set (intra-morph pollination 0.80 ± 0.03 ; self-pollination 0.82 ± 0.03) and seed production (intra-morph pollination, 204.40 ± 6.95 ; self-pollination, 211.70 ± 8.60).

Thousand-seed weight and germination rate

As shown in Table 1, the seed mass and vigor of the three morphs of *P. forbesii* were decreased to different degrees when seeds were obtained by self-pollination. For maternal L-morphs, the thousand-seed weight of intra-morph pollinations and self-pollinations were significantly reduced compared with $L \times S$ crosses ($F_{3,8} = 4.77$, $P = 0.03$), while the germination rate did not differ significantly among the different pollinations ($F_{3,8} = 1.45$, $P = 0.30$). In maternal S-morphs, there were no significant differences in the thousand-seed weight ($F_{3,8} = 3.20$, $P = 0.08$), while germination rates of seeds from intra-morph pollinations and self-pollinations, at only 62.67 ± 6.36 and $52.67 \pm 1.76\%$, respectively, were significantly lower than those of $S \times L$ and $S \times H$ seeds ($F_{3,8} = 14.34$, $P = 0.01$). For maternal H-morphs, self-pollination was associated with the lowest thousand-seed weight (87.33 ± 3.19 mg) and germination rate ($50.00 \pm 7.57\%$) among all 12 pollination types.

Inheritance of distyly and homostyly

Inheritance of distyly in *P. forbesii* was consistent with a standard Mendelian inheritance model (Table 2 and Fig. 5). The progeny of L-morph intra-morph pollinations and self-pollinations were all L-morphs. The floral morph frequency of progeny of S-morph intra-morph pollinations and self-pollinations did not deviate significantly from an L:S ratio of 1:3 ($S \times S$, $\chi^2 = 0.42$, $d.f. = 1$,

Table 1. The thousand-seed weight and germination rate of *Primula forbesii* seeds after artificial pollination.

| Maternal morph (♀) | Pollen sources (♂) | Weight of thousand seeds (mg) | Germination rate (%) |
|--------------------|--------------------|-------------------------------|----------------------|
| L-morph | S-morph | 114.40 ± 2.53 a | 88.67 ± 1.76 a |
| | H-morph | 106.93 ± 2.38 ab | 88.67 ± 2.40 a |
| | L-morph | 101.73 ± 2.45 b | 88.33 ± 1.76 a |
| | Selfing | 100.67 ± 3.87 b | 77.67 ± 8.35 a |
| S-morph | L-morph | 121.00 ± 1.89 a | 90.00 ± 3.46 a |
| | H-morph | 110.53 ± 1.95 a | 85.33 ± 5.81 a |
| | S-morph | 111.60 ± 4.00 a | 62.67 ± 6.36 b |
| | Selfing | 105.93 ± 5.15 a | 52.67 ± 1.76 b |
| H-morph | S-morph | 111.87 ± 3.16 a | 88.00 ± 2.00 a |
| | L-morph | 113.13 ± 3.10 a | 78.67 ± 6.57 a |
| | H-morph | 109.20 ± 3.35 a | 54.00 ± 3.46 b |
| | Selfing | 87.33 ± 3.19 b | 50.00 ± 7.57 b |

Means (s. e.) with different lowercase letters within a maternal morph are significantly different ($P < 0.05$) based on one-way analysis of variance.

$P = 0.56$; S-selfing, $\chi^2 = 0.13$, $d.f. = 1$, $P = 0.81$), although this frequency also did not deviate significantly from 1:2 ($S \times S$, $\chi^2 = 1.32$, $d.f. = 1$, $P = 0.28$; S-selfing, $\chi^2 = 1.80$, $d.f. = 1$, $P = 0.22$). Meanwhile, inter-morph pollinations yielded an L:S ratio of progeny that did not deviate from 1:1 ($S \times L$, $\chi^2 = 0.04$, $d.f. = 1$, $P = 0.92$; $L \times S$, $\chi^2 = 0.51$, $d.f. = 1$, $P = 0.54$). Moreover, for the progeny derived from outcrossing and selfing, the measurement of flower sex organs confirmed that there was an obvious bimodal pattern in stigma and anther height (Fig. 5).

Table 2. Flower morphs of *F*₁ progeny after artificial pollinations of L-morph, S-morph and H-morph *Primula forbesii*.

| Pollination type | L-morph | S-morph | H-morph | χ^2 value |
|--------------------------|---------|---------|---------|--------------------------|
| Deviation from L:S = 1:1 | | | | |
| S × L | 48 | 50 | – | 0.041 ns |
| L × S | 52 | 45 | – | 0.505 ns |
| Deviation from L:S = 1:3 | | | | Deviation from L:S = 1:2 |
| S × S | 27 | 70 | – | 0.416 ns |
| S-Selfing | 24 | 66 | – | 0.133 ns |
| L × L | 97 | – | – | – |
| L-Selfing | 100 | – | – | – |
| Deviation from L:S = 1:1 | | | | |
| S × H | 45 | 53 | – | 0.653 ns |
| H × S | 49 | 49 | – | 0 ns |
| L × H | 90 | – | 10 | – |
| H × L | 93 | – | 4 | – |
| H × H | 8 | – | 92 | – |
| H-Selfing | 2 | – | 93 | – |

Significant deviations from the predicted L:S ratio of 1:1, 1:2 or 1:3 was determined by χ^2 -test.

For seeds from selfing and outcrossing with other homostylous plants, almost all progeny were also homostylous plants. The progeny derived from crossing the H-morph and S-morph exhibited a roughly 1:1 floral ratio of L-morph and S-morph plants ($S \times H, \chi^2 = 0.65, d.f. = 1, P = 0.48$; $H \times S, \chi^2 = 0.65, d.f. = 1, P = 0.48$), while the progeny of crosses between the H-morph and L-morph were mainly comprised of L-morphs and a few H-morphs. Furthermore, for the progeny resulting from selfing and outcrossing of H-morphs, measurements of floral sex-organ position showed that there was substantial variation in stigma and anther height (e.g., Fig. 5I, L).

Progeny of open pollination

As shown in Table 3, the floral morph ratio of the cultivated population of *P. forbesii* was an L:S:H ratio of 0.52:0.40:0.08, in which the floral frequency of L-morphs and S-morphs significantly deviating from the expected L:S ratio of 1:1 ($\chi^2 = 4.94, d.f. = 1, P = 0.03$). For the L-morph seed families from open pollination, the progeny included L-morph, S-morph and H-morph plants, with an L:S ratio of 0.6:0.34, significantly deviating from 1:1 ($\chi^2 = 7.19, d.f. = 1, P = 0.01$). In the S-morph seed families, the progeny only included L-morph and S-morph plants, with an L:S ratio of 0.52:0.48, and the deviation from 1:1 was not significant ($\chi^2 = 0.16, d.f. = 1, P = 0.76$). In the H-morph seed families, many homostylous plants were observed, and the L:S:H floral morph ratio was 0.31:0.38:0.30.

DISCUSSION

The present study mainly focused on the inheritance of distyly and homostyly of *P. forbesii* and obtained the following key findings. First, confirming the results of previous research (Huu et al. 2022), the style length and heteromorphic incompatibility of the S-morph of *P. forbesii* could be changed by exogenous homo-BL, and thus, intra-morph pollination and self-pollination progeny of S-morphs can be obtained. Second, owing to inbreeding depression, the thousand-seed weight and germination rate of each floral morph decreased to different degrees after self-pollination. In addition, based on a 1:3:1:2 ratio of L-morph and S-morph progeny of intra-morph pollinations and self-pollinations of S-morphs, mature homozygous S-morphs (S/S) may experience viability selection. Third, based on the hemizygous inheritance model of *Primula*, distyly of *P. forbesii* is controlled by a single S-

locus, in which the L-morph is homozygous recessive (–/–) and the S-morph is hemizygous dominant (S/–). Consistent with Matsui et al. (2004), the homostyly may possess a genotype similar to that of the L-morph and arise via one or more unlinked modifier genes outside the S-locus. Finally, we determined that the autonomous self-pollination of L-morph and H-morph plants occurred frequently in the cultivated population, leading to the deviation from the equilibrium isocline of L-morphs and S-morphs. These key results of this study are discussed in detail below.

Exogenous homobrassinolide changes style length and female self-incompatibility

Self-incompatibility has been deeply studied in homomorphic plants, including sporophytic and gametophytic self-incompatibility (McCubbin 2008). In the former system, the pollen barrier site occurs on the stigmatic surface, while in the latter, it mainly occurs within the style (Allen and Hiscock 2008; Lu et al. 2018). For a heteromorphic incompatibility system, the incompatible pollen tube on the L-morph styles can partially enter the ovary but cannot germinate on the S-morph stigma (Huang et al. 2015; Jiang and Li 2017; Liu et al. 2022). For a long time, the female incompatibility of S-morphs had been considered entirely sporophytic, while such incompatibility was considered gametophytic in L-morphs (Lewis and Jones 1992; McCubbin 2008). Recently, Huu et al. (2022) provided a comprehensive and deep discussion on heteromorphic incompatibility in *P. forbesii*. They found that the gene *CYP734A50*, which encodes a brassinosteroid-inactivating enzyme, determined both style elongation and female self-incompatibility of S-morph plants. In the present study, consistent with previous results, by injecting exogenous homo-BL into S-morph flower buds, we successfully obtained 'long homostyly' and overcame self-incompatibility. Thus, the progeny of intra-morph pollination and self-pollination of S-morphs were successfully obtained.

Inbreeding depression and sheltered load

Under reproductive pressure caused by unreliable pollinator service and/or a limited number of mates, many strictly outcrossing species evolve self-compatibility (Stebbins 1974; Busch and Delph 2012; Igic and Busch 2013). Although the evolutionary transitions ensure the normal survival and reproduction of the population, it is commonly accompanied by strong inbreeding depression (Lande and

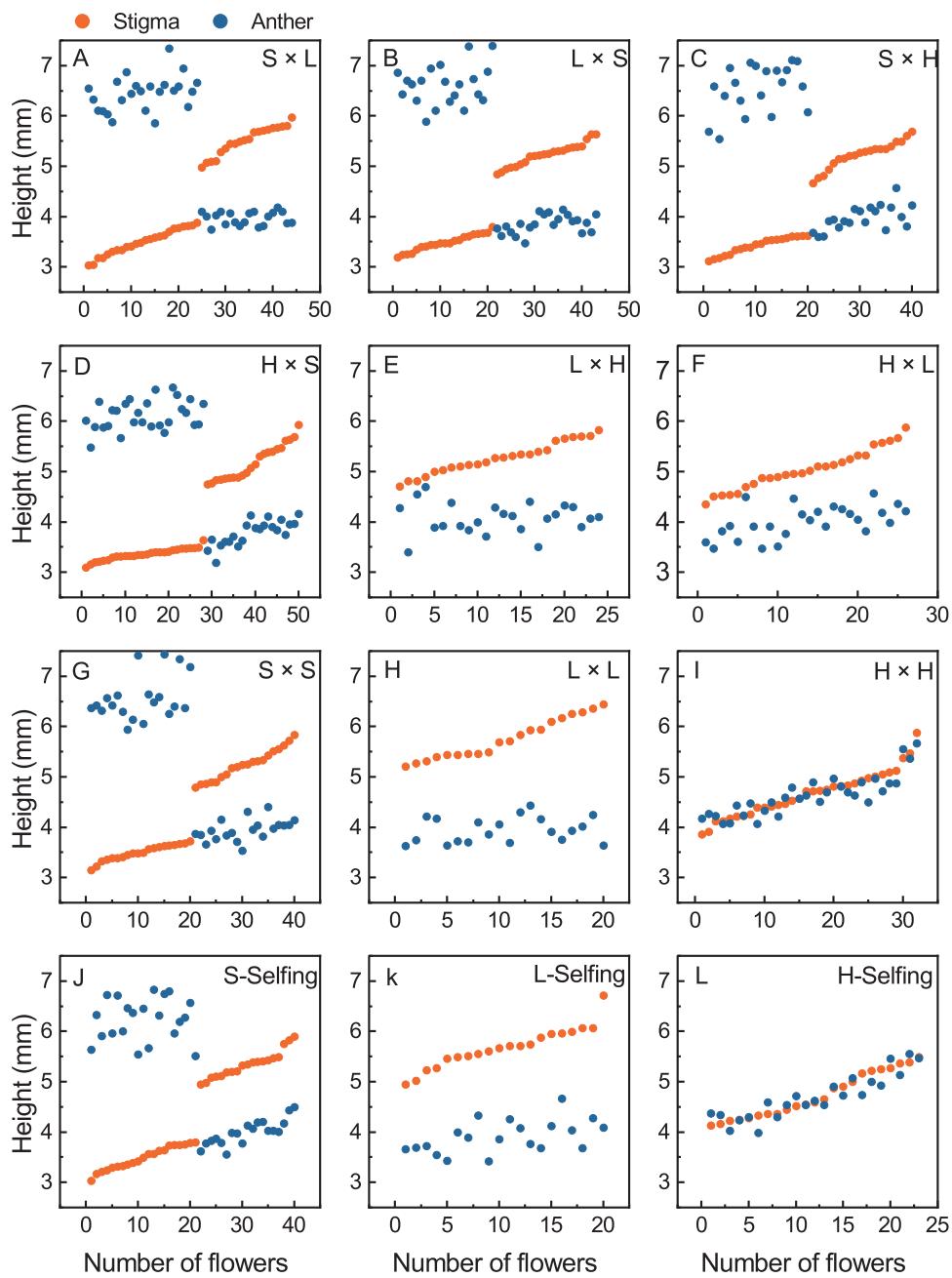


Fig. 5 Distributions of stigma and anther heights in the offspring generated by artificial pollination of *Primula forbesii* showed discrete patterns of variation. A S-morph \times L-morph; **B** L-morph \times S-morph; **C** S-morph \times H-morph; **D** H-morph \times S-morph; **E** L-morph \times H-morph; **F** H-morph \times L-morph; **G** S-morph \times S-morph outcross; **H** L-morph \times L-morph outcross; **I** H-morph \times H-morph outcross; **J** S-morph selfing; **K** L-morph selfing; **L** H-morph selfing.

Schemske 1985). Frequent self-fertilization in a population will result in increased homozygosity and the expression of recessive deleterious alleles, ultimately decreasing the fitness of progeny (Luijten et al. 2002; Charlesworth and Willis 2009; Barmentlo et al. 2018). Consistent with previous studies, we observed that the three floral morphs of *P. forbesii* exhibited declines in fitness after self-pollination, with seed masses and germination rates significantly lower than those of inter-morph pollination.

Owing to the forced hemizygosity of the *S*-locus in distylious species, it is postulated that the *S*-locus was tightly linked to one or more recessive deleterious alleles, a putative example of a phenomenon termed 'sheltered load' (Richards 1997; Strobeck 1972). According to this hypothesis, homozygous S-morphs (S/S)

would die or exhibit reduced viability in natural conditions. However, the empirical evidence for a 'sheltered load' associated with a *S*-locus are mixed (Mather and De Winton 1941; Barrett et al. 1989; Kurian and Richards 1997). In the present study, the L-morph to S-morph ratio was distributed between 1:3 and 1:2 in progeny of intra-morph pollinations and self-pollinations of S-morph plants. Consistent with Yuan et al. (2019), the mature homozygous S-morph plants may exist in progeny but also probably experience strong viability selection owing to linked loads.

Inheritance of distyly and homostyly

Heterostyly presents a classic model for the study of the shift from outcrossing to selfing. Hitherto, the inheritance of heterostyly has

Table 3. Floral morph ratio of the open-pollinated progeny of L-morph, S-morph and H-morph *Primula forbesii*.

| | L-morph | S-morph | H-morph | χ^2 value Deviation from 1 L:1 S |
|----------------------------------|-------------|------------|------------|---|
| Floral morph ratio of population | 157 0.52 | 120 0.4 | 23 0.08 | 4.942* |
| L-morph | 60 0.6 | 34 0.34 | 6 0.06 | 7.191* |
| | | | | |
| S-morph | 52 0.52 | 48 0.48 | – – | 0.16 ns |
| | | | | |
| H-morph | 31 0.31 | 38 0.38 | 30 0.3 | 0.71 ns |
| | | | | |

The χ^2 values with asterisks or "ns" correspond to significant or not significant deviations, respectively, from an L:S ratio of 1:1.

been investigated in c. 11 families, with the vast majority of studies focusing on distylous taxa (Lewis and Jones 1992). Distyly is controlled by a single *S*-locus linkage group, with two exceptions in which the dominance relations are reversed, with *S*-morph dominant heterozygotes (*S/s*) and *L*-morph recessive homozygotes (*s/s*) (Charlesworth and Charlesworth 2009; Barrett 2019). Based on molecular data, the *S*-haplotype of the *S*-morph actually contains a hemizygous region consisting of at least five genes, which is absent from the *s*-haplotype of *L*-morphs (Li et al. 2016; Kappel et al. 2017). The hemizygous characteristics of the *S*-locus have been demonstrated in *Primula*, *Turnera*, *Fagopyrum*, and *Linum* (Yasui et al. 2012; Li et al. 2016; Shore et al. 2019; Gutiérrez-Valencia et al. 2022). In the present study, the results of different cross experiments are consistent with previous inheritance models, indicating that the *S*-morph is dominant hemizygous (*S/–*) and the *L*-morph is recessive homozygote (*–/–*) in *P. forbesii*. It is unsurprising that numerous *Primula* species share the same inheritance pattern, because phylogenetic analysis of *Primula* has demonstrated a single origination of distyly (Mast et al. 2006).

Self-compatible homostyly occurs frequently in distylous populations under reproductive pressure. Homostylos flowers commonly combine the sex organs of distyly, in which long homostyly occurs as a chimera of the styles of *L*-morphs and anthers of *S*-morphs, and short homostyly exhibits the opposite combination (Barrett and Shore 2008). Therefore, homostyly for a long time had been considered to originate from homologous recombination within the *S*-locus region (Pamela and Dowrick 1956; Lewis and Jones 1992). However, this hypothesis has now been rejected after the dominant *S*-haplotype was demonstrated to be hemizygous, because the *s*-haplotype could not provide homologous sequences with which to pair and cross-over during meiosis (Li et al. 2016; Kappel et al. 2017). Homostyly probably has arose via mutations of the key genes controlling either style length or anther height within the *S*-locus, which has been supported by molecular data from many distylous species (Huu et al. 2016; Shore et al. 2019; Huu et al. 2020). However, Mora-Carrera et al. (2023) found in *P. vulgaris* that the *S*-morph and some long-homostylos plants share exactly the same *CYP* allele, which could inhibit style elongation by inactivating brassinosteroid and thus produce short styles. These results suggested that in addition to loss-of-function mutation of the coding region of specific genes, homostyly may also arise via other substitution mechanisms, such as promoter region mutations, structural rearrangements and mutations to unlinked modifier genes outside the *S*-locus (Mather and De Winton 1941; Ganders 1979; Kappel et al. 2017). In the present study, the progeny of intra-morph pollination and self-pollination of homostylos plants were almost all homostylos, indicating that the homostylos variants of

P. forbesii were homozygous. Consistent with the findings of Matsui et al. (2004), no or only a few homostylos plants were observed to result from cross experiments of homostylos plants with *S*-morphs and *L*-morphs, indicating that homostyly could not originate from the mutations of key genes within the *S*-locus. Based on crossing data alone, we speculate that homostylos plants may have a genotype similar to that of *L*-morphs (*–/–*) and have arisen via one or more recessive modifier genes outside the *S*-locus. There were a few homostylos plants among the progeny of crosses between homostylos plants and *L*-morphs, which may indicate that some heterozygous *L*-morphs provide recessive modifier genes. However, it is still difficult to explain why there were a small number of *L*-morphs among the progeny of intra-morph pollinations and self-pollinations of homostylos plants. Environmental factors may play a role in the expression of polygenic modifiers based on the considerable fluctuation of sex-organ height in progeny. Thus, it will be necessary to sequence the genes that control the style length and anthers height (e.g., *CYP734A50* and *GLO2*), which will provide direct evidences for the origination and inheritance of homostyly in *P. forbesii*.

Mating patterns in open-pollinated conditions

Heteromorphic incompatibility has great significance in maintaining the disassortative mating and equilibrium isocline of floral morphs in heterostylos species (Ganders 1979; Brys and Jacquemyn 2015; Yuan et al. 2017; Yuan et al. 2019). For distylous species partially or completely lacking heteromorphic incompatibility, the distribution of progeny for each floral morph after open pollination is an effective method to evaluate mating style, although more quantitative evaluation requires the use of molecular markers (Barrett and Shore 1987; Yuan et al. 2019). In previous studies, the absence of heteromorphic incompatibility was shown to seriously disrupt the disassortative mating system and lead to a deviation from a 1:1 ratio of *L*-morphs and *S*-morphs (Barrett 2003; Yuan et al. 2017; Shao et al. 2019; Mora-Carrera et al. 2023). In the cultivated population of *P. forbesii*, the open-pollinated progeny of *S*-morphs did not different from a 1:1 morph ratio. However, open-pollinated *L*-morphs apparently produced more progeny of *L*-morph, about twice the number of *S*-morphs. This pattern is likely caused by frequent occurrences of intra-morph fertilization and self-fertilization in *L*-morph plants owing to the weakened heteromorphic incompatibility. Owing to the loss of heteromorphic incompatibility and herkogamy, homostylos plants would have a higher incidence of autonomous self-pollination compared with distylous plants. In the present study, nearly 1/3 of the open-pollinated progeny of homostylos plants were homostylos. The open-pollination results accord with the inheritance pattern described above, as self-pollination of homostylos plants would produce exclusively homostylos plants, indicating that autonomous self-pollination occurs frequently in homostylos plants. Furthermore, the biased morph ratio of the cultivated population of *P. forbesii* corresponded well with above results, indicating the frequent occurrence of self-pollination in *L*-morph and homostylos plants in the open pollination conditions. Therefore, theory can predict, although the direct evidence remains lacking (Curtis and Curtis 1985), that *S*-morphs may be further reduced and ultimately result in a homostylos population with a few *L*-morph plants (Crosby 1949; Crosby 1960; Mora-Carrera et al. 2023). Studies on large time scales are necessary, which will provide direct evidence to elucidate the effects of shifts in mating systems on floral morph composition and evolutionary fate of heteromorphic populations.

DATA AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper.

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AUTHOR CONTRIBUTIONS

YJ, CL, and YP planned and designed the research. CL, YL, YX, and ZW performed the experiments. XY and QL analyzed the data. CL, YJ, and SG wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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