



A novel type of mirror-image flowers caused by lateral bending of the floral tube in a bumblebee-pollinated plant

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ABSTRACT

We studied a previously unreported form of mirror-image flowers in *Rhabdothamnopsis sinensis* (Gesneriaceae) endemic to China. The style in *R. sinensis* remains straight throughout the blooming period, while the basal part of the floral tube bends either to the left or right side, causing the style to lean towards the right or left side of the floral tube. *R. sinensis* has two epipetalous fertile stamens with twisted filaments, moving the fused anthers located at the opposite side of the style. This floral syndrome results in reciprocal mirror-image flowers in a relative simpler way as compared to typical types of mirror-image flowers. Pollinator observations revealed that the bending of the floral tube and twisted filament block the entrance to the floral tube, rendering long-tongued bumblebees as pollinators by transferring pollen with the side of the thorax. *R. sinensis* is self-compatible and the pollen-ovule ratio is strikingly low (37.41 ± 11.90). Thorax pollination probably is the main reason for such low pollen wastage because the thorax suffers less grooming than the abdomen, which is the main pollen-carrying part in other typical mirror-image flowers. The unusual type of mirror-image flowers described here suggests a specialized pollination adaptation with limited but high-fidelity pollinators.

1. Introduction

Mirror-image flowers are a highly-specialized sexual polymorphism in which the style deflects either to the left or right side of a flower in the same species (Todd, 1882; Barrett et al., 2000; Jesson and Barrett, 2002). Depending on whether or not there are fertile stamen(s) deflecting to the opposite side of the style, mirror-image flowers can be classified as reciprocal or nonreciprocal mirror-image flowers (Jesson and Barrett, 2002). Mirror-image flowers can be expressed at two fundamentally different patterns of organization, i.e. the left- and right-styled flowers can be produced either on the same individual (monomorphic enantiostyly) or on different individuals (dimorphic enantiostyly) (Jesson and Barrett, 2002, 2003).

Typical mirror-image flowers have no obvious floral tube and sexual organs are presented to pollinators for buzz-pollination (pollinators vibrate poricidal anthers to release pollen) (Todd, 1882; Jesson and Barrett 2002, 2005). Normally, mirror-image flowers are pollinated by large-bodied insects such as bumblebees or carpenter bees (Müller, 1883; Jesson and Barrett, 2002, 2003). These insects can touch the

deflected style and stamen(s) respectively with their left or right side and consequently facilitate pollen transfer between left- and right-styled flowers (Fenster, 1995; Jesson and Barrett, 2002, 2005; Lin and Tan, 2007). Therefore, mirror-image flowers can increase the precision of cross-pollen transfer (Jesson and Barrett, 2002) and the spatial separation of the deflected style and stamen (herkogamy) is crucial for touching the left or right side of the pollinator's body, and is the key trait for successful pollen pickup and deposition (Ren et al., 2013).

As a medium-size pantropical family, Gesneriaceae is well known for the high occurrence and diversity of mirror-image flowers (Harrison et al., 1999; Lu et al., 2019). Mirror-image flowers in Gesneriaceae are restricted to the Old World subfamily Didymocarpoideae, and are known from about 80 species from seven genera (Lu et al., 2019). In contrast to most angiosperms, mirror-image flowers in Didymocarpoideae are mainly nonreciprocal, with several distinct floral traits such as united anthers and an obvious floral tube (Harrison et al., 1999; Lu et al., 2019), suggesting a specialized pollination mechanism and distinct evolutionary histories of mirror-image flowers in this subfamily.

Here, we report a previously undescribed type of mirror-image

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flowers in *Rhabdothamnopsis sinensis* Hemsley, a perennial herb of Didymocarpoideae from Southwest China (Wang et al., 1998; Möller et al., 2016), the center of diversification and endemism of the subfamily (Li and Wang, 2005; Wei, 2010). This species is unique for its laterally bending floral tube, causing the style to locate at the left or right side of the floral tube and resulting in mirror-image flowers within the same individual (monomorphic enatiostyly). As far as we know, this novel type of mirror-image flower has not yet been reported in angiosperms.

In a natural population near Kunming City, Yunnan Province, Southwest China, we studied the floral biology, floral morphometrics, and pollination ecology of *Rhabdothamnopsis sinensis* to answer the following questions. (1) What type of mirror-image flower (reciprocal versus nonreciprocal) occur in *R. sinensis*? (2) What are the floral traits, pollination mechanism and breeding system of *R. sinensis*? (3) What is the implication of this novel type of mirror-image flowers for floral evolution in Gesneriaceae?

2. Materials and methods

2.1. Species and study site

Rhabdothamnopsis sinensis Hemsley is a perennial, non-rhizomatous subshrub. Stems are simple or branch from the base, with opposite leaves. Leaves are sometimes crowded near the stem apex. Flowers are solitary, axillary, with a peduncle of 1–3 cm, 1–2 bracts, and a 5-lobed calyx. The corolla is purple to purple-white, with purple and yellow stripes on the low lobes. The species is known from the Yunnan, Guizhou, Sichuan and Hunan provinces of Southwest China (Wang et al., 1998; Xiao et al., 2015). *R. sinensis* is narrowly distributed in limestone mountains and probably is the only species of the genus, although sometimes another variant (*R. sinensis* var. *ochroleuca* W. W. Smith) is recognized (Wang et al., 1998; Li and Wang, 2005).

R. sinensis grows mainly on dry limestone rocks in forest or along forest edges (Wang et al., 1998). Our fieldwork was conducted during the peak flowering season in July and August of 2018 in a population on West Mountain of Kunming City (24°58'3"N, 102°37'37"E, 2176 m a.s.l.). The population consists of more than 100 individuals of *R. sinensis*. Main coexisting plants are *Leptodermis potanini* (Rubiaceae), *Stellaria media* (Caryophyllaceae), *Oxalis pescaprae* (Oxalidaceae), *Fagopyrum esculentum* (Polygonaceae) and some species of Cyperaceae and Poaceae.

2.2. Floral traits

To determine the floral phenology and quantify the production of left- and right-styled flowers, we counted the number and ratio of left- and right-styled flowers at both individual plant and population level (two to six inflorescences per plant). To determine the floral biology and blooming pattern of *R. sinensis*, 10 ~ 20 randomly selected individuals with flower buds were marked and observed from 8:00 to 9:00, 12:00 to 13:00 and 17:00 to 18:00 h each day, until the flowers were completely wilted (normally five days later). The floral size (width of floral mouth), floral tube length, and herkogamy (anther-stigma separation) at the vertical and horizontal directions were measured with digital calipers to the nearest millimeter during the anthesis period (Fig. 1).

2.3. Flower visitors and behaviors

We monitored the types and behavior of flower visitors between 08:00–18:00 h during three consecutive days in August of 2018. Each flower visitor was photographed while the pollinator entered the flower, in order to record the types of visitors and their visiting frequencies (visits•flower⁻¹•h⁻¹). We recorded and compared behavior and visiting frequency of the main flower visitors. To assess whether a mechanical fit exists between mirror-image flowers and flower visitors, we captured several individuals of the main visitors with a net and measured body size (thorax width, abdomen width with tongue length) with digital

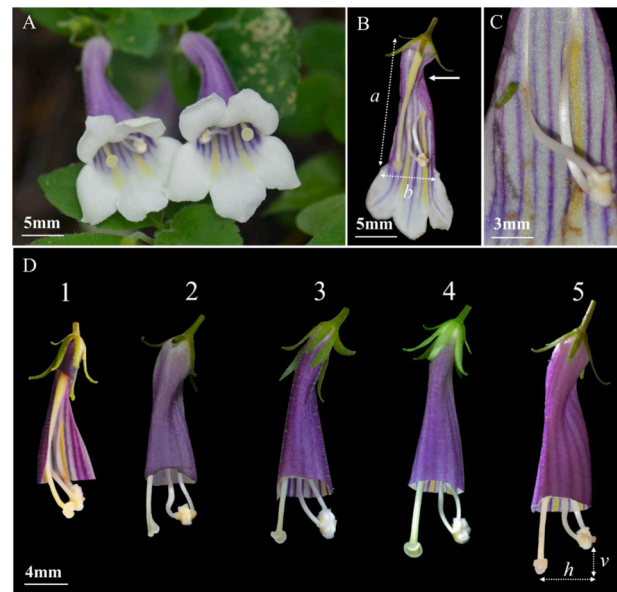


Fig. 1. Mirror-image flowers and floral syndromes of *Rhabdothamnopsis sinensis*. A, Left- and right-styled flowers on the same branch. B, Style deflection was mainly achieved via side-bending of floral tube (arrow), the length of the floral tube (a) and width of floral mouth (b) are shown. C, Filament twisting moved the united anthers to the opposite side of the deflected style. D, The developmental stages of mirror-image flowers, note that both horizontal (h) and vertical (v) separations of anthers and stigma (herkogamy) are presented when flowers mature.

calipers to the nearest millimeter for comparing with herkogamy (horizontal) and floral tube length. We also dyed pollen grains in anthers of ~ 80 open flowers with 1% safranin solution. Two or three individuals for each type of floral visitors were captured after they visited a flower, using two electric mosquito swatters. These captured insects were checked under a stereomicroscope to determine where on the body of the visitor the dyed pollen was deposited. The floral visitors with dyed pollen (red) on their bodies were determined as legitimate pollinators.

2.4. Pollen-ovule ratio

Twenty fresh flower buds from different individuals were collected and fixed in FAA (70% ethanol: formaldehyde solution: acetic acid = 90:5:5) to count pollen number and ovule number for the calculation of pollen-ovule (P/O) ratios. Anthers in each flower bud were removed in cellulase solution (1%) for 5 h to soften the pollen sac and release pollen grains (Dafni, 1992). Three separate 5 µl subsamples were transferred onto slides by using a pipette and pollen grains were counted using a stereomicroscope (100 ×). Similarly, we dissected ovaries in each flower bud to count the number of ovules.

2.5. Outcrossing index

According to Dafni (1992), the outcrossing index was calculated by the sum of flower size (0, 1, 2, 3 for a flower diameter of < 1, 1–2, 2–6, > 6 mm, respectively), herkogamy (1, 0 for herkogamy or not), and dichogamy (1, 0 for protandry or not). A high value of the outcrossing index normally indicates a higher degree of outcrossing and more dependence on pollinators (Dafni, 1992). The outcrossing index is used here as a rough approximation of the mating system together with hand-pollination experiments.

2.6. Pollen viability and stigma receptivity

To test pollen viability and stigma receptivity and their lifespan, 30

flowers during anthesis from different developmental stages (0, 1, 2, 3, 4, 5 days after flower opening, five for each) were collected at 10:00–12:00 h. Pollen viability was assessed by means of 15% ($\text{g}\cdot\text{ml}^{-1}$) sucrose solution. Pollen from the same developmental stage was mixed into the sucrose solution for about two hours. Three random samples with at least 20 pollen grains per sample were examined under a stereomicroscope (100 \times). Pollen grains that produced obvious pollen tubes were considered viable. Following the method of Dafni (1992), stigma receptivity was measured with 3% ($\text{g}\cdot\text{ml}^{-1}$) hydrogen peroxide (H_2O_2), and a stigma that releases obvious oxygen bubbles is considered receptive.

2.7. Hand-pollination experiments

We used 10 individual experimental plants to determine self-compatibility and the ability of autonomous self-pollination in *R. sinensis*. Hand-pollination experiments were conducted and compared with open pollination during July and August of 2018. We isolated flowers in showerproof bags to verify the occurrence of fertilization under several hand-pollination experiments. Five pollination treatments were carried out on the 10 plants. Each treatment was performed using 10 buds. All treatments were carried out on the first day of flower opening, and pollinators were excluded from all flowers until wilting, apart from the open-pollination treatment. To test for spontaneous self-pollination, flowers were left unmanipulated; to test for apomixes, flowers were emasculated by careful removal of anthers; to test whether plants are self-compatible, flowers were hand-pollinated with pollen from their own anthers and compared to flowers that were cross-pollinated with pollen from a different plant; an open-pollinated control treatment was also included.

Fruit set and seed set of each treatment was counted two weeks later when fruits were mature (fruits became brown). We calculated the autofertility index (average fruit set of spontaneous self-pollination divided by average fruit set of cross pollination) according to Eckert et al. (2010) to measure the level of autonomous self-fertilization.

2.8. Data analysis

To test whether pollinator and floral dimensions differed, we analyzed body size (including the thorax breadth and beak length) of three kinds of visitors and floral traits (including the length of floral tube and herkogamy) using a Least Significant Difference (LSD) analysis in SPSS 17.0 (IBM, Chicago, IL, USA).

For the main types of floral visitors, their visit frequencies were compared to determine whether there are differences in visitation frequency of these visitors, using independent-samples *t*-tests. Fruit set was analysed using generalized linear models implemented in SPSS 17.0 (IBM, Chicago, IL, USA). To account for potential correlations in fruit set among flowers on the same plant, we used Generalized Estimating Equations (GEE) in which 10 plants were treated as the subject variables. An exchangeable correlation matrix was incorporated in models and significance was assessed using Wald statistics. For analyses of the proportion of flowers that set fruit, we used the binary logistic model. Additionally, the proportion of fruit set among different pollination treatments was compared using the Dunn-Sidak-procedure. The analysis was performed in SPSS 17.0. For graphing of the mean proportions and standard errors, we back-transformed marginal means from the logit scale, resulting in asymmetrical error bars.

3. Results

3.1. Floral traits

Flowers of *Rhabdothamnopsis sinensis* usually start opening in the morning before 11:00, and last for 4.01 ± 1.04 d (mean \pm standard deviation). *R. sinensis* produces 2–6 axillary inflorescences with one

flower in each inflorescence (solitary). The floral tube is purple but the five lobes were white. The upper lip is 2-lobed, while the lower lip is 3-lobed (Fig. 1A). The inner wall of the floral tube contains ~ 10 purple stripes (Fig. 1). The style is straight or slightly curved (Fig. 1B) and two fertile stamens are anther-coherent (Fig. 1). Flowers of *R. sinensis* are hermaphroditic, with the stigma becoming receptive on day 1 of anthesis (Fig. 2B). The anthers dehisce via longitudinal slits ~ 1 h after the flower opens (Fig. 1).

The length of floral tube in the mature flower is 33.54 ± 1.39 mm (mean \pm standard deviation, $n = 10$) and the width of floral mouth is 8.04 ± 0.87 mm ($n = 10$). The horizontal herkogamy (horizontal separation of anthers and the stigma) of the left- and right-styled flowers is 4.79 ± 1.31 mm and 4.75 ± 0.57 mm (mean \pm standard deviation, $n = 10$), respectively. The vertical herkogamy of the left- and right-styled flowers is 2.61 ± 0.69 mm and 2.71 ± 0.72 mm, respectively (Fig. 2A). The horizontal herkogamy remains unchanged during anthesis, while the vertical anther-stigma separation increases slightly during flower development, resulting in a final stigma location at 3.45 ± 0.44 mm ($n = 10$) above the anther position. There is no significant difference in horizontal and vertical herkogamy between left- and right-styled flowers ($t = 0.059$, $P = 0.955$; $t = 0.199$, $P = 0.847$) (Fig. 2A).

The basal part of the floral tube in *R. sinensis* bends obviously to the left or right side during bud development (Fig. 1B, D), causing the style to lean towards the right or left side of the floral tube. Simultaneously, the filaments of two fertile stamens are inserted into different position on the floral tube (Fig. 1C) and the filaments are curved to the same direction of floral tube bending. As a result, stigma and anthers are reciprocally positioned at opposite sides of the floral tube (Fig. 1A, B), resulting into reciprocal mirror-image flowers. *R. sinensis* inflorescences bear left- and right-styled flowers, so this species exhibits monomorphic enantiostyly at the individual plant level. The ratio of left- to right-styled flowers is approximately 1:1 at both the individual plant (1.91 vs. 1.82; $n = 127$ flowers of 34 individuals) and the population (135 vs. 144; $n = 279$ flowers of 126 individuals) level.

3.2. Pollen-ovule ratio and pollen and stigma viabilities

Ovules and pollen production per flower are 1240 ± 137 ($n = 20$) and $45,567 \pm 12,817$ ($n = 20$) respectively. Therefore, the pollen-ovule ratio (P/O ratio) is 37.41 ± 11.90 . The outcrossing index of *R. sinensis* was 4, according to the method of Dafni (1992).

The sucrose solution test (Fig. 2B) suggests that pollen viability is low ($< 34.79\%$) just after flower opening and during day 1 of anthesis, then raises to $70.43 \pm 12.63\%$ at the day 2 of anthesis, but drops ($< 25.56\%$) after day 3 of anthesis and is only $4.64 \pm 3.55\%$ at the last day of anthesis. The stigma receptivity, however, shows a very high value just after flower opening and could last for three to four days (Fig. 2B).

3.3. Floral visitors

Most frequent visitors to *R. sinensis* flowers are *Lasioglossum* sp. and two species of *Bombus* (*Bombus* sp.1 = 1.32 ± 1.05 visits \cdot flower $^{-1}\cdot$ h $^{-1}$ ($n = 132$), *Lasioglossum* sp. = 1.51 ± 0.73 visits \cdot flower $^{-1}\cdot$ h $^{-1}$ ($n = 151$), and *Bombus* sp.2 = 0.02 ± 0.04 visits \cdot flower $^{-1}\cdot$ h $^{-1}$ ($n = 2$)) (Table 1). There is no significant difference for *Bombus* sp.1 and *Lasioglossum* sp. in visitation rate ($t = 1.131$, $P = 0.280$), but the visitation frequency of both *Bombus* sp.1 and *Lasioglossum* sp. were significantly higher than that of *Bombus* sp.2 ($t = 6.275$, $P = 0.001$) (Table 1). *Lasioglossum* sp. landed on the lower floral lip, and crept along the floral tube in search of pollen (Fig. 3B). *Lasioglossum* sp. got dusted with pollen grains mainly on their ventral surface but they rarely touched the stigma. *Bombus* sp.1 also landed on lower floral lip, but moved along the nectar guides (purple and yellow stripes in the inner wall of the floral tube) to forage in the floral tube for nectar (Fig. 3A). Due to the filaments lying across the floral tube, *Bombus* sp.1 cannot enter deeply and its thorax is situated at the floral mouth and near the deflected style and anthers (Fig. 3).

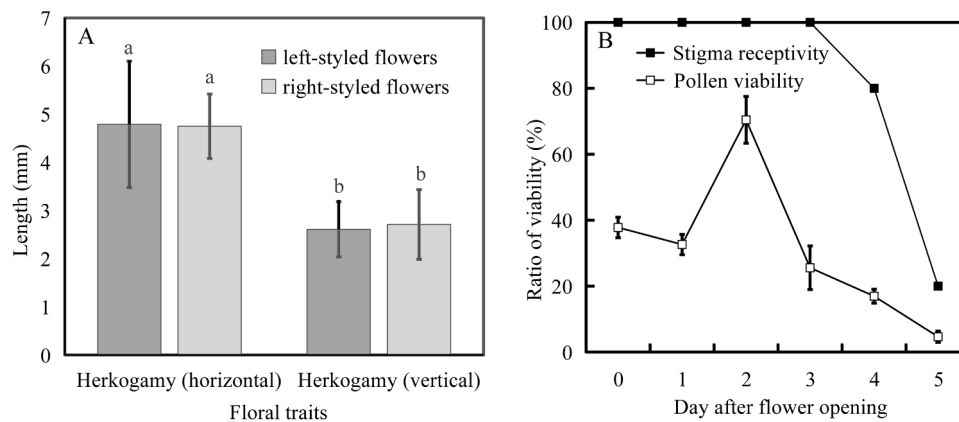


Fig. 2. Changes of horizontal and vertical herkogamy (anther-stigma separation) (A) and pollen viability and stigma receptivity (B) of *Rhabdothamnopsis sinensis* during the anthesis period (mean \pm SD, $n = 10$). Different letters above bars indicate significant difference at $P < 0.01$.

Table 1
Main floral visitors and their visit frequencies of *Rhabdothamnopsis sinensis*.

Floral visitor	Body size (mm) (Abdomen width)	Body length (mm) (with tongue)	Visit frequency (visits \cdot flower $^{-1}\cdot$ h $^{-1}$) (No. of visits)
<i>Bombus</i> sp.1	4.98 \pm 0.20 ^a	22.55 \pm 0.98 ^a	1.32 \pm 1.05 ^a (132)
<i>Lasioglossum</i> sp.	2.23 \pm 0.17 ^b	7.54 \pm 0.14 ^b	1.51 \pm 0.73 ^b (151)
<i>Bombus</i> sp.2	4.81 \pm 0.27 ^a	17.94 \pm 0.33 ^c	0.02 \pm 0.04 ^b (2)

*Different letters indicate significant difference at $P < 0.005$.

Therefore, when *Bombus* sp.1 forages for nectar with its long tongue, the powdery pollen grains are easily released and adhered to its thorax sides (Fig. 3C, D). Meanwhile, the stigma can touch the other side of the thorax of *Bombus* sp.1. The foraging behavior of *Bombus* sp.2 was similar to that of *Bombus* sp.1, but visitation frequency of *Bombus* sp.2 was lower (Table 1).

The body size (thorax breadth) of *Bombus* sp.1, *Bombus* sp.2 and *Lasioglossum* sp. were 4.98 \pm 0.20 mm ($n = 9$) and 4.81 \pm 0.14 mm ($n = 5$) and 2.23 \pm 0.17 mm ($n = 9$) respectively (Table 1). The horizontal herkogamy (horizontal distance between anthers and stigma) was 5.04 \pm 0.30 mm ($n = 9$) and matches well with body sizes of both bumblebees, *Bombus* sp.1 ($P > 0.05$) and *Bombus* sp.2 ($P > 0.05$), all are significantly wider than the body size of *Lasioglossum* sp. ($P < 0.001$, Fig. 4A) (one-way ANOVA on horizontal herkogamy and body sizes: $F_{3,28} = 44.708$, $P < 0.001$). The body length with tongue of *Bombus* sp.1,

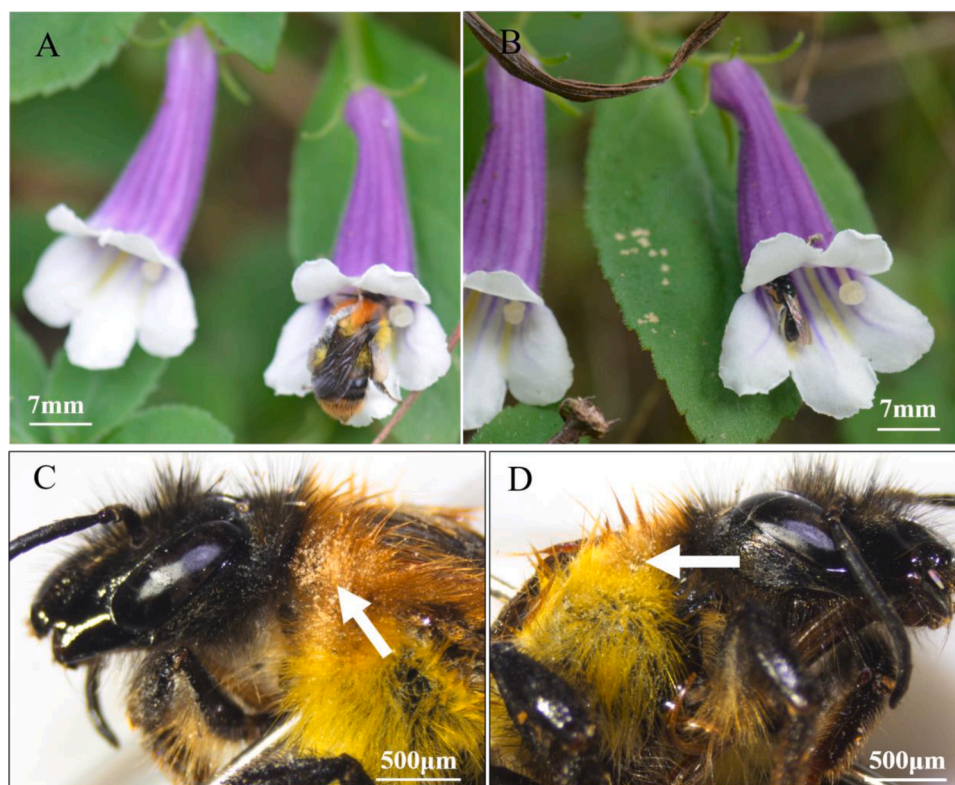


Fig. 3. The most frequent floral visitors are *Bombus* sp.1 (A) and *Lasioglossum* sp. (B). Pollen grains can be carried by left (C) and right (D) sides of the thorax of *Bombus* sp.1.

Bombus sp.2 and *Lasioglossum* sp. were 22.55 ± 0.98 mm ($n = 5$), 17.94 ± 0.33 mm ($n = 5$) and 7.54 ± 0.14 mm ($n = 9$) (Table 1). The floral tube length was 21.31 ± 1.04 mm ($n = 10$) and matches well with body length (with tongue) of *Bombus* sp.1 ($P > 0.05$), both are significantly longer than body lengths of *Bombus* sp.2 ($P < 0.001$) and *Lasioglossum* sp. ($P < 0.001$, Fig. 4B) (one-way ANOVA on floral tube length and body lengths: $F_{3,25} = 78.79$, $P < 0.001$).

3.4. Hand-pollination

Ovaries of the emasculated and bagged flowers did not expand, therefore this treatment was excluded from the statistical analysis of pollination treatments. There was a significant effect of treatment on the proportion of fruit set ($\chi^2 = 10.890$, $df = 3$, $P < 0.05$). The proportion of flowers that set fruit following self-pollination and cross-pollination was higher than that of flowers that received open pollination. The proportion of flowers that set fruit following spontaneous self-pollination was lower than that of flowers that received open pollination. In addition, the pairwise comparisons between different treatments showed that the proportion of fruit set following self-pollination and cross-pollination differed significantly from that resulting from spontaneous self-pollination (Dunn's test: $P = 0.005$) (Fig. 5). Results of the hand-pollination experiments revealed that *R. sinensis* is largely self-compatible. The autofertility index was 0.4972. No apomixis was revealed (Fig. 5). The species experienced pollen limitation in the wild since artificial cross-pollination resulted in higher fruit set than open-pollination (Fig. 5).

4. Discussion

Typical mirror-image flowers have no obvious floral tube and sexual organs are presented to pollinators for buzz-pollination (Todd, 1882; Jesson and Barrett 2002, 2005). If a floral tube is present in mirror-image flowers, it is normally straight, shallow and wide and pollinator can easily reach to the bottom of the floral tube (Gao et al., 2006; Lin and Tan, 2007). Mirror-image flowers in *Rhabdothamnopsis sinensis*, however, are distinct from the typical form because of a laterally bending floral tube, twisting filaments, and longitudinally-dehiscent anthers. In contrast to typical mirror-image flowers adapted to insects

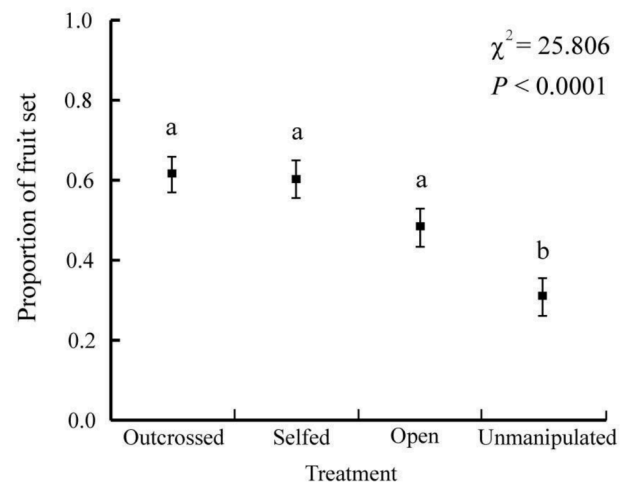


Fig. 5. Mean (\pm SE) fruit set of *Rhabdothamnopsis sinensis* flowers subjected to different pollination treatments ($n = 10$). Unmanipulated stands for the treatment in which flowers were bagged without treatment.

carrying pollen on their abdomens, pollen of *R. sinensis* is transferred on the lateral sides of the bumblebee thorax, suggesting a distinctive pollination mechanisms and floral evolution in this narrowly-distributed plant.

4.1. Why does the floral tube bend laterally?

Although floral tube bending (e.g. sigmoid corollas) (Guo and Wang, 2014) was previously reported in Gesneriaceae, the lateral bending of the floral tube in *Rhabdothamnopsis sinensis* described here may be unique in flowering plants. We propose that, compared to the style deflection in typical mirror-image flowers, the unique lateral bending of the floral tube in *R. sinensis* probably has two adaptive functions. Firstly, the flower not only has the style at the side of the floral tube but also simultaneously moves the epipetalous stamens to the opposite side of the style (Fig. 1B, C). This particular floral architecture may have experienced relatively less developmental constraint during its

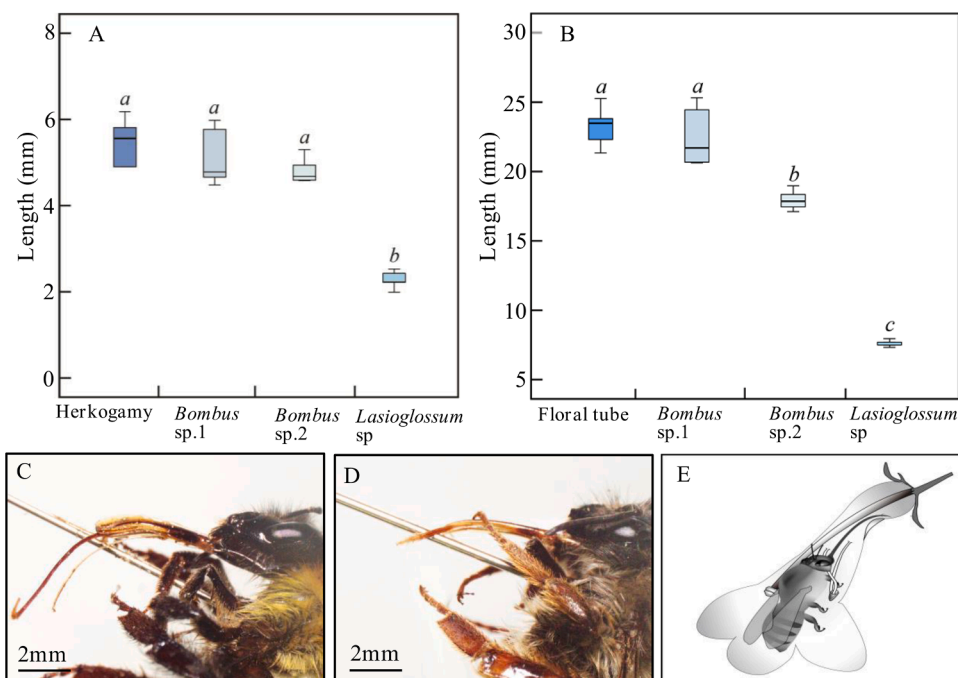


Fig. 4. A, Herkogamy (horizontal) and the body size (thorax breadth) of main floral visitors (mean \pm SD, $n = 10$). Different lowercase letters indicate significant differences at $P < 0.001$. Body length (with tongue) of main visitors (B) (data are mean \pm SD, $n = 5$), different letters indicate significant differences at $P < 0.001$. The tongue of *Bombus* sp.1 (C) is longer than *Bombus* sp.2 (D). E, Mechanical fit between floral traits and the main pollinator *Bombus* sp.1, note that the bumblebee touches stigma and anthers with its thorax sides.

Table 2
Comparison of pollen-ovule ratio (P/O) for species in Gesneriaceae.

Species	Main floral traits	Pollinator	P/O	Reference
<i>Rhabdothamnopsis sinensis</i>	Mirror-image flowers (reciprocal)	<i>Bombus</i> sp.	37.41 ± 11.9	This study
<i>Ornithoboea wildenian</i>	Mirror-image flowers (nonreciprocal)	<i>Lasioglossum</i> sp.	253.7 ± 94.1	Lu and Ren, unpublished
<i>Oreocharis auricula</i>	Free anthers	<i>Bombus</i> sp.	250.61 ± 83.17	Zhang, 2011
<i>Oreocharis pumila</i>	Sigmoid corolla, Anther-united	<i>Nomia</i> sp.	295 ± 125	Guo and Wang, 2014
<i>Oreocharis acaulis</i>	Approach herkogamy, Anther-united	<i>Bombus</i> sp.	677 ± 341	Guo et al., 2013
<i>Raphiocarpus macrosiphon</i>	Long and narrow floral tube	<i>Nomia</i> sp., <i>Amegilla</i> sp.	53.37 ± 11.19	Zhang et al., 2010
<i>Beccarinda argentea</i>	Anther-united	<i>Bombus</i> sp.	665.39 ± 172.45	Zhang et al., 2010
<i>Hemiboea subcapitata</i>	Anther-united, style movement	<i>Amegilla</i> sp., <i>Bombus</i> sp.	157.79 ± 14.98	Zhang, 2011
<i>Hemiboea cavaleriei</i>	Anther-united, filament movement	<i>Amegilla</i> sp.	241.28 ± 38.17	Zhang, 2011
<i>Primulina cordata</i>	Anther-united	<i>Trigona ventralis</i>	331.76 ± 13.08	Huang et al., 2016
<i>Primulina lutea</i>	Anther-united	<i>Apidae</i> sp., <i>Crabro</i> sp., <i>Amegilla</i> sp.	1215.73 ± 266.13	Tang et al., 2009
<i>Primulina gueilinensis</i>	Anther-united	<i>Chalcididae</i> sp., <i>Amegilla albigena</i>	1918.10 ± 519.25	Pu et al., 2009

evolutionary history than the typical reciprocal mirror-image flowers, in which the style and the fertile stamen(s) must respectively deflect to the opposite direction to gain the reciprocal placement of two sexual organs (Jesson and Barrett, 2002, 2003; Ren et al., 2013; Lin and Tan, 2007). Second, the extremely narrow floral tube caused by the lateral bending of the tube may have further restricted the nectar foraging of floral visitors and thus only specialized long-tongued bees are legitimate pollinator (Fig. 1C, Fig. 4C). In a closely related species *Didissandra macrosiphon* (Hance) W. T. Wang, the floral tube is straight and suffers severe nectar robbing from the generalized pollinator *Nomia* sp. (Zhang, 2011). The obstructions caused by the lateral bending and contracted floral tube of *R. sinensis* can also prolong the visitation time of a pollinator, increasing contact of the pollinators with anthers and consequently promoting greater pollen pickup. Similarly, in *Oreocharis pumila* (Gesneriaceae), a species with a sigmoid corolla (the floral tube bends vertically), prolonged pollinator visit duration and enhanced pollen removal also occurs (Guo and Wang, 2014). These findings are consistent with the view that the shape of the floral tube selects for long-tongued or long-billed pollinators and functions to restrict their foraging behavior and thus plays a key role in pollination by increasing pollen transfer precision (Darwin, 1877; Fenster, 1991; Silva and Sazima, 1995).

4.2. Why are filaments twisted?

The twisted filaments lie across the floral tube and may restrict the probing of bumblebees into the base of the floral tube (Fig. 4C). This might serve as a mechanism, together with the narrow part of the laterally-bending floral tube, to prevent bumblebees from entering and keeping their thorax at the floral mouth, where the stigma and anthers are located. Such floral traits only allow long-tongued insects to reach the nectar at the bottom of a floral tube. The twisted filaments can also prolong the visitation time and increase contact frequency between the insect thoraxes and sexual organs, and consequently enhance pollen export (Fig. 4). Similar mechanisms involving filament structures have also been reported in the lever-like stamens in *Salvia* L. (Claußen-Bockhoff et al., 2004; Zhang et al., 2010) and the filament curtain in Acanthaceae (Manktelo, 2000).

4.3. Why such low pollen-ovule ratios?

The extraordinary low P/O ratio in *R. sinensis* is hard to explain, but the low P/O ratio is also found in another Gesneriaceae species with mirror-image flowers, i.e. *Ornithoboea wildenian* Craib (Table 2) and in other species with specialized pollination systems (Jürgens et al., 2002; Chouteau et al., 2006; Lozada-Gobilard et al., 2019). Some studies have found that the P/O ratio is negatively correlated with pollen transfer efficiency (Shaanker and Ganeshaiah, 1984; Harder and Johnson, 2008;

Gong and Huang, 2014; De Jager and Joubert, 2016). Therefore, the extraordinary low P/O ratio in *R. sinensis* may indicate a relatively high pollen transfer efficiency in this species, which has also been reported for several other taxa with mirror-image flowers (Jesson and Barrett, 2002, 2005; Ren et al., 2013).

The hypothesized high pollen transfer efficiency in *R. sinensis* is probably achieved mainly through its specialized floral traits associated with the long-tongued bumblebees (Fig. 4). In this species, pollen is transferred on the sides of the thorax of bumblebees (Figs. 3, 4). Compared to insect abdomens that typical mirror-image flowers use for pollen transfer, the thorax suffers less grooming and is a safer place to carry pollen grains. As a consequence, pollen wastage may be decreased and could explain the low P/O ratio.

4.4. Suggestion for further study

Two alternative evolutionary hypotheses can be proposed to explain the development of the unusual mirror-image flowers in *Rhabdothamnopsis*. (i) The filaments became twisted firstly in *Rhabdothamnopsis*, similar to *Kaisupeeia cyanea*, and then subsequently the floral tube evolved to be longer and narrower to increase pollination precision. Finally the lateral bending of the floral tube was selected through visitation by specialized long-tongued bumblebees. (ii) First, *Rhabdothamnopsis* has a long but straight floral tube as its ancestral condition. A mutation causing floral bending occurs subsequently and the filaments become twisted to promote reciprocal placement of the two sexual organs thus enhancing the precision of pollen transfer. To test these alternative hypotheses, studies involving evo-devo approaches in closely related genera are needed to explore the evolutionary sequences and adaptive basis of key floral traits associated with the unusual mirror-image flowers of *R. sinensis*.

CRediT authorship contribution statement

Shao-Jun Ling: Formal analysis, Investigation, Methodology, Project administration, Resources, Writing - original draft. **Tao Lu:** Conceptualization, Data curation, Writing - original draft. **Ming-Xun Ren:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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