

Different Morphs of Heterostylous Plant (*Tirpitzia Sinensis*) Associated With Floral Characters Have Various Adaptation Strategies to Pollinator to Ensure Reproductive Success

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Research article

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Abstract

Background: Heterostylous plants are commonly associated with pollinators promoting disassortative pollination. How different morphs adapt to pollinators to ensure reproductive success and whether floral characters (such as pistil, stamen and nectar traits) are relevant to this process remain unclear.

Results: *Tirpitzia sinensis* is distylous flowers. Its floral characters were measured. Field observation of effective pollinator and pollination efficiency to different morphs was conducted, and breeding systems were examined by hand pollination treatments. Our results showed that L-morph produced more but smaller pollen grains per anther than S-morph. *T. sinensis* secreted more nectar at night as a possible adaptation to hawkmoths (*Macroglossum* spp.) active at dusk. L-morph produced much nectar due to its large secretion volume at night. The nectar was rich in sucrose. The sucrose/hexose ratio was higher in the nectar of S-morph than in that of L-morph. S-morph had higher visit rate but lower pollination efficiency of hawkmoths compared with L-morph. *T. sinensis* was self-incompatible. Seed sets in nature did not differ between morphs.

Conclusions: Our findings suggest that L-morph increases its pollination efficiency through outstanding stigma, many pollen grains per anther, and high amount of nectar. S-morph attracts many hawkmoths to pollinate due to its high sucrose/hexose ratio in its nectar.

Background

Heterostyly exhibits variation and complexity in the positions of floral sexual organs, including distyly and tristyly [1–3]. Distyly is recorded from nearly 28 angiosperm families and accounts for 1–2% of flowering plants, such as Primulaceae, Boraginaceae, Plumbaginaceae, Polemoniaceae, Polygonaceae and Linaceae [4]. Heterostyly has exhibited evolutionary importance in avoiding self-pollination, promoting cross-pollination and reducing interference between male and female functions in the same flower [1, 3, 5].

Heterostyly usually occurs in narrow tubular flowers but rarely in open bowl-shaped flowers [3, 6]. In addition to reciprocal herkogamy, the stamen characters (such as number and size of pollen grains, ornamentation of pollen exine, anther size and colour), stigma depth and areas of heterostyly also show diversity and are generally referred to as ancillary polymorphism [7–9]. Long-styled morphs (hereafter L-morphs, low anthers) usually produce more but smaller pollen grains than short-styled morph (S-morphs, high anthers) [7, 8, 10]. The stigma of L-morphs is usually larger than that of S-morphs [11–13]. Distylous plants are usually self-incompatible [7, 14].

Nectar is generally present and concealed at the base of floral tubes in distylous plants. Long-tongued pollinators descend into the floral tube to seek nectar, thus transferring pollen [3, 6]. The nectar characteristics of L- and S-morphs could affect the pollinator's behaviour [15]. These two morphs have similar nectar sugar concentration with rich glucose and fructose in *Fagopyrum esculentum*. S-morphs secrete more nectar with higher proportion of sucrose than L-morphs. Honeybee spends long time on S-

morphs and often visits S-morph inflorescences [15]. To our knowledge, comparison on the floral characters, especially nectar traits (nectar volume, sugar concentration and nectar composition) between the two morphs of distyly plants and how they adapt to effective pollinators have not been studied.

Hawkmoths are important pollinators found in some warm areas at dusk that fly forward with a fast wing beat and feed from flowers with exceptionally long tongues while hovering [16]. Hawkmoth pollination has been reported in a wide range of angiosperm taxa [17–19]. *Tirpitzia sinensis* (Linaceae) is an odourless distylous plant with white narrow tubular flowers. Whether hawkmoths pollinate narrow tubular distylous plants has been barely reported. The different adaptation strategies of the L- and S-morphs of *T. sinensis* to pollinators to ensure reproductive success remain unclear.

This study aimed to (1) compare floral characters, especially the nectar volume, sugar concentration and composition, between L- and S-morphs; (2) detect the effective pollinators of *T. sinensis* and compare the visit rate and pollination efficiency (pollen removal and receipt of one visit) between L- and S-morphs; and (3) test whether *T. sinensis* is self-incompatible and compare the natural seed production between L- and S-morphs.

Results

Difference in floral characters between the two morphs

The leaves were relatively larger in the S-morph than in the L-morph (marginal significant difference, see Table 1). The S-morph has larger sepal, petal width, floral tube depth, stamen length and pollen size than the L-morph (all $P < 0.05$). The flower length, petal length, pistil length, anther length, pollen grain number and pollen/ovule ratio of L-morph were larger than those of S-morph (all $P < 0.05$). Other floral traits including flower width, opening diameter, anther width, anther thick, single flowering days and ovule number were not significantly different between the two morphs (all $P > 0.05$) (Table 1). Generally, L-morph had longer pistil, produced more pollen grains per flower and consequently had higher pollen/ovule ratios than S-morph. Meanwhile, S-morph had longer stamen and larger pollen than L-morph.

Table 1

Comparisons of vegetative and reproductive traits (mean \pm SE) between long-styled morphs (hereafter L-morph) and short-styled (hereafter S-morph) morph of *Tirpitzia sinensis* tested by a generalized linear model (GLM) analysis. Values of one morph significantly larger than the other are written in bold.

	L-morph	S-morph	Wald χ^2	df	P
Leaf length(mm)	45.98 \pm 0.57	49.45 \pm 0.96	3.890	1	0.049
Leaf width(mm)	26.67 \pm 0.57	28.25 \pm 0.55	3.981	1	0.046
Sepal length(mm)	6.32 \pm 0.12	7.03 \pm 0.12	17.836	1	< 0.001
Sepal width(mm)	2.87 \pm 0.04	3.02 \pm 0.04	7.045	1	0.008
Flower length(mm)	23.64 \pm 0.28	22.49 \pm 0.28	8.482	1	0.004
Flower width(mm)	22.29 \pm 0.27	21.63 \pm 0.27	2.934	1	0.087
Petal length(mm)	11.62 \pm 0.14	11.23 \pm 0.14	4.056	1	0.044
Petal width(mm)	9.11 \pm 0.15	9.77 \pm 0.16	9.455	1	0.002
Opening diameter(mm)	1.49 \pm 0.03	1.47 \pm 0.02	0.368	1	0.544
Tube depth(mm)	32.19 \pm 0.26	35.05 \pm 0.29	54.607	1	< 0.001
Tube diameter(mm)	1.86 \pm 0.03	1.87 \pm 0.02	0.098	1	0.754
Pistil length(mm)	36.26 \pm 0.29	30.64 \pm 0.33	164.13	1	< 0.001
Stamen length(mm)	29.94 \pm 0.26	36.98 \pm 0.29	334.767	1	< 0.001
Anther length(mm)	1.81 \pm 0.06	1.31 \pm 0.03	50.254	1	< 0.001
Anther width(mm)	0.56 \pm 0.02	0.53 \pm 0.02	2.517	1	0.113
Anther thick(mm)	0.36 \pm 0.01	0.35 \pm 0.01	0.071	1	0.790
Single flowering(d)	3.39 \pm 0.07	3.53 \pm 0.11	1.124	1	0.289
Pollen grain number	3187.94 \pm 120.80	1268.21 \pm 51.30	70.485	1	< 0.001
Pollen length(μ m)	45.87 \pm 0.56	68.86 \pm 0.93	445.653	1	< 0.001
Pollen width(μ m)	46.02 \pm 0.62	69.04 \pm 0.85	483.332	1	< 0.001
Ovule number	8.18 \pm 0.13	8.21 \pm 0.12	0.026	1	0.871
Pollen/ovule ratio	394.42 \pm 17.47	154.41 \pm 6.44	183.970	1	< 0.001

Measurement of nectar volume, sugar concentration and properties

T. sinensis produced significantly ($P < 0.001$, Wald $\chi^2 = 47.197$, df = 1) larger volume (μ l) nectar at night (2.83 ± 0.15) than during the day time (1.50 ± 0.13). The sugar concentration (%) of nectar secreted at

night (16.51 ± 0.56) and during the day time (14.96 ± 0.71) had no significant difference ($P = 0.086$, $\text{Wald}\chi^2 = 2.955$, $\text{df} = 1$) (Fig. 1B). Moreover, L-morph produced significantly ($P = 0.038$, $\text{Wald}\chi^2 = 4.302$, $\text{df} = 1$) more nectar (3.13 ± 0.23) than S-morph (2.53 ± 0.16) at night. During the day time, the nectar volume produced by L- (1.65 ± 0.20) and S-morphs (1.35 ± 0.15) had no significant difference ($P = 0.234$, $\text{Wald}\chi^2 = 1.419$, $\text{df} = 1$). The nectar sugar concentration produced by both morphs at night and during the day time had no significant difference (all $P > 0.05$) (Fig. 1B).

Fructose, glucose and sucrose were detected in *T. sinensis* nectar through HPLC analysis. The sucrose contents (9.15 ± 0.65) in nectar (all nectar property data of L- and S-morphs combined together and analysed) were significantly higher ($P < 0.001$, $\text{Wald}\chi^2 = 51.145$, $\text{df} = 2$) than the fructose (4.75 ± 0.34) and glucose contents (5.96 ± 0.37). The nectar sugar properties of L- and S-morphs had no significant difference ($P = 0.212$, $\text{Wald}\chi^2 = 1.556$, $\text{df} = 1$). For the nectar of the L-morph, the sucrose contents were significantly higher than the fructose contents, and glucose contents had no significant difference with sucrose and fructose contents ($P = 0.005$, $\text{Wald}\chi^2 = 10.538$, $\text{df} = 2$) (Fig. 1C). For the nectar of the S-morph, the sucrose contents were significantly higher than the fructose and glucose contents, and the fructose and glucose contents had no significant difference ($P < 0.001$, $\text{Wald}\chi^2 = 48.048$, $\text{df} = 2$) (Fig. 1C). Baker and Baker (1983) conducted a sucrose to hexose ratio (r) assessment, where r equals to amounts of sucrose / (amounts of glucose + amounts of fructose). The nectar of L-morph of *T. sinensis* was rich in sucrose because the r equals to 0.696, $8.5 / (6.7 + 5.5)$, and the nectar of S-morph of *T. sinensis* was sucrose dominant, $r = 1.1$, $10 / (3.8 + 5.0)$. The sucrose/hexose ratio was higher for S-morph flowers than for L-morph flowers.

Pollinator species and abundance

Hawkmoth (*Macroglossum*), bumblebees (*Bombus*) and honeybees (*Apis*) were the major floral visitors of *T. sinensis* through the field observation in 2018 and 2019. The long-tongued hawkmoths probed the nectar at the base through the narrow floral tube. In this process, the pollen was removed and deposited onto the tongue, which was then stained with pollen and could touch the stigma to achieve effective pollination (Fig. 2A, B). The bumblebees always only robbed the nectar (Fig. 2C), and the honeybees mainly groomed the *T. sinensis* pollen into their corbiculae (Fig. 2D). We also found that *Macroglossum* frequently pollinated the *T. sinensis* flowers at 18:00–20:30 but seldom during the rest of the day.

The visit rates of hawkmoths (0.81 ± 0.20), bumblebees (0.60 ± 0.09) and honeybees (0.37 ± 0.06) to *T. sinensis* had no significant difference ($P = 0.247$, $\text{Wald}\chi^2 = 2.797$, $\text{df} = 2$). The visit rates of bumblebees, honeybees and hawkmoths were not significantly different for the S-morph ($P = 3.687$, $\text{Wald}\chi^2 = 0.158$, $\text{df} = 2$) and for the L-morph ($P = 0.706$, $\text{Wald}\chi^2 = 0.695$, $\text{df} = 2$). However, the visit rate of hawkmoths to S-morph (1.19 ± 0.35) was significantly higher ($P = 0.030$, $\text{Wald}\chi^2 = 4.683$, $\text{df} = 1$) than that to L-morph (0.35 ± 0.08) (Fig. 3).

Pollen transfer efficiency of hawkmoths

The pollen removal and receipt of hawkmoth in L-morph was significantly higher than that in S-morph (see Table 2).

Table 2

Comparisons of pollen efficiency (pollen removal and pollen receipt of one visit by hawkmoth) (mean \pm SE, sample size) between the L- and S-morphs of *Tirpitzia sinensis* analyzed by GLM. Values of L-morph significantly larger than those of S-morph are written in bold.

	L-morph	S-morph	Wald χ^2	df	P
Pollen removal	2774.60 \pm 73.62 (48)	994.33 \pm 37.25 (46)	418.602	1	< 0.001
Pollen receipt	66.35 \pm 11.97 (48)	9.91 \pm 1.78 (46)	51.408	1	< 0.001

Breeding system of *T. sinensis*

T. sinensis had a cryptic self-incompatibility system because nearly no seeds were produced (seed set % 1.44 ± 0.89 , $n = 60$. Data of L- and S-morph pollination treatments combined together and analysed) following intramorph pollination. By contrast, the seed set (%) resulting from intermorph pollination was significantly high (43.47 ± 5.45 , $n = 60$). The seed set of L-morph as pollen recipients (19.16 ± 2.54) was not significantly different from that of S-morph (11.65 ± 2.01) (Table 3). For the L-morph, the fruit set of intermorph pollination (62.17 ± 7.23 , $n = 30$) was significantly higher than that of the control treatment (38.19 ± 4.85 , $n = 30$), intramorph pollination treatment (0.00 ± 0.00 , $n = 30$), self-pollination treatments (1.63 ± 1.63 , $n = 30$), autogamy treatments (2.17 ± 2.17 , $n = 30$) and emasculated treatments (0.00 ± 0.00 , $n = 30$) ($P < 0.001$, Wald $\chi^2 = 234.255$, $df = 5$) (Fig. 4). For the S-morph, the fruit set of intermorph pollination (28.45 ± 6.82 , $n = 30$) was not significantly different from that of control treatment (37.27 ± 7.78 , $n = 30$), but was significantly higher than that of intramorph pollination treatments (2.59 ± 1.57 , $n = 30$), self-pollination treatment (7.76 ± 3.58 , $n = 30$), autogamy treatments (0.00 ± 0.00 , $n = 30$) and emasculated treatments (0.00 ± 0.00 , $n = 30$) ($P < 0.001$, Wald $\chi^2 = 75.451$, $df = 5$) (Fig. 4). Significant interactions were found between pollen recipient morph and pollination treatments with respect to seed set (Table 3). Moreover, the seed set under control treatment had no significant difference between L- and S-morphs ($P = 0.999$, Wald $\chi^2 = 0.00$, $df = 1$).

Table 3

Generalized linear model: effect of pollen recipient morph (L- and S-morphs) and pollination treatments (control, intermorph, intramorph, self-, autogamy and emasculated) and their interaction on seed set (%) in *Tirpitzia sinensis*.

Source of variation	df	wald χ^2	P
Pollen recipient morph	1	0.965	0.326
Pollination treatments	5	238.863	<0.001
Interaction	5	23.962	<0.001

Discussion

Hawkmoth flowers produce copious nectar to support the large energy requirement of the visitor. The nectar is usually diluted to ensure that it flows easily through the thin proboscis [20–22]. The *Petunia axillaris* were pollinated by hawkmoths, and the nectar volume was high (approximately 35 μ l), but the sugar concentration (approximately 16%) was low [23]. The *Agrius convolvuli* pollinated by long-tongued hawkmoths produced higher volume (approximately 15 μ l) and dilute (sugar concentration approximately 27%) nectar [24]. Similarly, the nectar sugar concentration of *T. sinensis* was low. Although one flower secreted a small amount of nectar, the plant still has numerous flowers.

Flowers may control their nectar and thus affect pollinator's behaviours [21]. By manipulating the nectar volume of *Mirabilis multiflora*, Hodges [25] showed that hawkmoths always visit e flowers on plants with a large amount of nectar. The amount of nectar produced by mutational *Petunia integrifolia* individuals per flower was only 1/3 of that produced by wild-type individuals; hawkmoth pollinators reduced the probing duration on low-nectar plants when they are exposed simultaneously to low-nectar and wild-type *P. integrifolia* [23]. The nectar volume of *T. sinensis* secreted during the night was higher than that secreted during the day, a possible adaptation strategy to *Macroglossum* pollinator flying at dusk. L-morph secreted more nectar than S-morph and could increase the probing duration of hawkmoths and consequently increase the pollination efficiency. The nectar composition could potentially act as an important agent for visitor activities. Hummingbirds, hawkmoths and long-tongued bees prefer sucrose-rich nectar, and short-tongued bees and flies prefer hexose (glucose and fructose)-rich nectar [15, 21]. The nectar of hawkmoth-pollinated *Petunia axillaris* was also rich in sucrose (approximately 57% in nectar sugar proportion) [23]. The nectar of buckwheat pollinated by honeybees is rich in glucose and fructose[15]. The nectar of *T. sinensis* is rich in sucrose as an adaptation to hawkmoth pollination.

The natural seed production between the different morphs of distylous plants always has no significant difference to maintain the balance between the numbers of L- and S-morphs in one population, such as in *Pentanisia* species (Rubiaceae) and *Fagopyrum esculentum* (Polygonaceae) [15, 26, 27]. The seed set of the L- and S-morphs of *T. sinensis* did not differ significantly. The seed set with the control treatment of L-morph was significantly lower than that with intermorph pollination treatment. The seed set with control

treatment and intermorph pollination treatment of S-morph had no significant difference. These results indicated that the L-morph of *T. sinensis* exhibited a legal pollen (intermorph pollen) limitation may due to the low visit rate of hawkmoth to this morph type.

Heterostyly promotes compatible pollination between floral morphs within species since Darwin[1]. Therefore, the legal and illegal pollens on the stigma of L- and S-morphs must be detected to test the above hypothesis. Most heterostylous species are tubular flowers (such as *Tirpitzia sinensis*), and some are bowl-shaped flowers. However, the reproductive strategies of the two types of corolla formations in heterostylous plants remain unknown. The tubular flower *T. sinensis* is pollinated by hawkmoths (*Macroglossum*), and the bowl-shaped flower *Linum suffruticosum* is pollinated by bee-flies (Bombyliidae)[28]. We plan to explore whether and how distylous plants with different corolla shapes promote compatible pollination by using two species in Linaceae disturbed in China including *Tirpitzia sinensis* (tubular flower) and *Linum usitatissimum* (open bowl-shaped flower).

Conclusion

The distylous *T. sinensis* was effectively pollinated by hawkmoths (*Macroglossum*). The S-morph had higher visit rate of hawkmoths but lower pollination efficiency of hawkmoths compared with the L-morph. The visitor *Bombus* always robbed the nectar, and *Apis* gathered the pollen as pollen thieves. *T. sinensis* produced nearly no seeds with intramorph pollination, and the natural seed production of both morphs had no significant difference. The long floral tube and nectar traits (large amount of nectar secreted during the night, low nectar sugar concentration and high sucrose contents in the nectar) of *T. sinensis* may be used to adapt to hawkmoth pollination. Moreover, the outstanding stigma, high pollen amount per anther and large amount of nectar of L-morph could be employed to adapt to the pollinators and increase the pollination effectiveness of hawkmoths to L-morph. L-morph stigmas are usually accessible to pollinator [7, 13, 27]. The high sucrose/hexose ratio in the nectar of S-morph might attract many hawkmoths to visit the inflorescence.

Methods

Study species and sites

T. sinensis Hemsley (Linaceae) is a shrub or tree mainly distributed in Guangxi, Guizhou, Yunnan (southeast) of China at an elevation of 300–2000 m and usually grows in calcareous soil on mountain slopes or along trails. Plants can be 1–5 m tall with blade elliptic, obovate–elliptic or ovate leaf. The cymes inflorescence is generally 4 cm terminal or axillary at the top of stem or branch. Each flower consists of five green sepals and five white petals arranged into floral tubes, and the nectar is usually present and concealed at the base of the floral tube. Five stamens surround the central four pistils. Flowers are homogamy and usually last 3–4 days. Plants usually flower from May to August. Capsules contain three to eight seeds and mature 3 months after fertilisation [29]. Our field investigation of pollination ecology revealed that *T. sinensis* is a typical distylous plant with L-morph (anthers are placed

low in the corolla, and stigmas are located high) and S-morph (anthers are placed high, and stigmas are located low) (Figure 1A) in the field populations located in Laoshan Provincial Nature Reserve (104°49' 62" E, 23°94' 8" N, approximately 1700 m above sea level), Malipo country, Yunnan province, southwest China.

The plant materials in this research are obtained under the permission of Laoshan Nature Reserve Bureau, Yunnan province, China. The formal identification of the plant is undertaken by Liu Changqiu, associate researcher, Guangxi Institute of Botany, Chinese Academy of Sciences. A voucher specimen photo of *T. sinensis* has been deposited in Plant Photo Bank of China (PPBC), the deposition number is xyc74220920100731.

Difference in traits between the L- and S-morphs of *T. sinensis*

To compare plant performance between the two morphs, we randomly selected 50 plants (each plant selected one flower) per morph and measured two vegetative and thirteen reproductive traits, including leaf length and width; sepal length and width; flower length, width and opening diameter; tube depth; petal length and width; stamen length; pistil length; and anther length, width and thickness to 0.01 mm using a caliper micrometre.

To compare pollen, ovule production and pollen size, we selected 30 flower buds from 30 L-morph individuals and 30 S-morph individuals respectively and stored them in a 1.5 ml centrifuge tube filled with 75% alcohol for fixation and preservation. The anther and ovary from one flower were separated using forceps in the laboratory, and the anthers were suspended in 500 ml of water. Three drops (each drop of 50 ml) of every pollen solution sample were counted under the Nikon E100 optical microscope. The mean of the three pollen drops was multiplied by 10 to estimate the pollen production of one flower. The ovules were counted under a stereomicroscope. The P/O ratio was equal to the number of pollen grains divided by the corresponding ovule number. For pollen size estimation, three pollen grains per flower were first photographed, and the length and width were then measured using Digimizer Version 4.6.0. To compare the single flower period between the two morphs, we marked one bud of the above selected 30 individuals each for L- and S-morph and recorded the first day of the opening state. Every 2 days, we recorded the flower opening state until the anthers and pistil lost function. These days were denoted as the single flower period.

Measurements of nectar volume, sugar concentration and properties of *T. sinensis*

To compare the nectar volume and concentration in *T. sinensis* during anthesis between day and night, we bagged and labelled 30 flowers before anthesis from 30 plants each from L- and S-morphs. During the male flowering phases, the nectar in the bagged flower was removed using glass microcapillary tube (0.3 mm in diameter) on the day before the measurement. Nectar was extracted from the flowers bagged from 18:30 to 06:30 (secreted during the night). After the treatments, the same flower was bagged again, and the nectar was extracted from 06:30 to 18:30 (secreted during the day) the next day. The length (L) of the microcapillary tube occupied by nectar was measured using a caliper micrometre. The volume (V_{total}) and

length (L_{total}) of one standard microcapillary were calculated, and the volume of nectar (V) is equal to $L/L_{\text{total}} * V_{\text{total}}$. And the concentration of nectar was measured with a hand-held refractometer (Eclipse 0%–50%; Bellingham and Stanley Ltd., Basingstoke, United Kingdom; see [30]).

To measure sugar components, we collected nectar from control-bagged flowers of L-morph (30 flowers from 30 individuals) and S-morph (27 flowers from 27 individuals) of *T. sinensis* by using microcapillary tubes. After the nectar length was measured using a caliper micrometre, the nectar was spotted onto filter paper and was air-dried at room temperature [31]. The spotted filter papers were placed in a 1.5 ml centrifuge tube and stored in the refrigerator at $-20\text{ }^{\circ}\text{C}$. The sugars were removed by elution with $100\text{ }\mu\text{l}$ of deionised water at room temperature for 24 h. Sugar type (glucose, fructose, sucrose and maltose) was identified, and the relative mass was quantified by High Performance Liquid Chromatography (HPLC, Waters Corporation, Milford, Massachusetts) with a refractive index detector and an Agilent Zorbax carbohydrate analysis column 843300-908 (Agilent Technologies, Santa Clara, California) under the column temperature of $35\text{ }^{\circ}\text{C}$. The mobile phase was 80% acetonitrile, the flow rate was 1 ml/min and the injection volume was $20\text{ }\mu\text{l}$. Quantities of each sugar in nectar samples were determined by the standards (glucose, fructose, sucrose and maltose) using the regression equations (based on response peak areas to standard sugar mass) and were expressed as relative percentage by mass [32].

Pollinator species and abundance

To determine the pollinator species of *T. sinensis*, we observed all visits of different species over 2018 and 2019 in several populations with three or four individuals including hundreds of flowers. Visitor observation of L- and S-morphs lasted for 9 sunny days (July 15, 16, 17, 19, 21, 22, 23, 24, 26 and 29) in 2018 and 8 sunny days (June 22, 26, 27, 28, 29, 30 and July 1, 3) in 2019. Each session lasted for 30 minutes between 7:00 to 22:00, and the visitor's observations of L- and S-morph were conducted simultaneously. We randomly selected 10 populations containing both L- and S-morph individuals and completed 40 and 44 sessions in 2018 and 2019, respectively. Visitor moves in one population were recorded to quantify visitation rates to L- and S-morphs. Visit number per foraging, visitor species and foraging behaviour were recorded, and the total opening flowers in each population were counted. The visit frequency of one visitor was expressed as the number of visits per flower per hour.

Pollen transfer efficiency of hawkmoths

To compare the pollination efficiency of hawkmoth between the L- and S-morphs, we estimated the pollen removal and receipt per morph. Male-phase inflorescence (previously unvisited) were bagged until anther dehiscence, and each inflorescence was allowed a single visit by a hawkmoth. To estimate pollen removal, we collected 48 visited flowers for L-morph and 46 visited flowers for S-morph from different plants with another 48 L-morph buds and 46 S-morph buds as the control. Each flower was stored in a 2 ml centrifuge tube with 75% alcohol. Pollen removal per flower was calculated from the mean number of pollen grains in unvisited flowers minus the mean number of pollen grains remaining after one visit. To estimate pollen receipt per visit, we removed undehisced anthers from the 48 male-phase flowers for L-

morph and 46 male-phase flowers for S-morph and bagged these flowers with cotton mesh until they developed into the female phase. These female-phase inflorescences were then removed from the bag and allowed one visit by the hawkmoth. Stigmas of these visited emasculated flowers were collected and stored in a 1.5 ml centrifuge tube with alcohol. Pollen grains from the anthers and on the stigmas were counted under a light microscope (Nikon E100). The anthers were fully mashed with tweezers to form 0.5 ml of pollen suspension. Three drops (each drop of 50 μ l) of every pollen solution sample were counted, and the mean was multiplied by 10 to estimate pollen production (for undehisced anthers) or pollen remaining per flower (one single visited anther) [33].

Breeding system

To determine whether *T. sinensis* is self- and intramorph incompatible, we conducted artificial pollination experiments as follows: (1) open pollination as control; (2) intramorph pollination (L-morph as pollen receptor and received L-morph pollen from other individuals and S-morph as pollen receptor and received S-morph pollen from other individuals); (3) intermorph pollination (L-morph as pollen receptor and received S-morph pollen from other individuals and S-morph as pollen receptor and received L-morph pollen from other individuals); (4) self-pollination (pollen from the flowers in the same individuals); (5) autogamy treatments (the flowers were bagged all the time without any treatments); and (6) emasculated treatments. In 30 individuals each for L- and S-morphs, six flowers were marked with a cotton thread of different colours. Four of the six flowers were emasculated and bagged until they developed into the female phase and then received intramorph, intermorph, self- and emasculated pollination treatments. The remaining two flowers were used as the control and autogamy pollination treatments. Three months after pollination, seeds per flower of six pollination treatments were collected and counted.

Data analysis

To assess the differences in plant performance between L- and S-morphs, we compared 15 plant vegetative and reproductive traits, single flowering days and P/O (pollen number/ ovule number) using a generalized linear model (GLM) with normal distribution and identity-link function. The pollen number and ovule number between L- and S-morphs were compared using Poisson distribution with loglinear-link function in GLM (all plant characters as dependent variable, and L- and S-morphs as factors). Nectar volume and sugar concentration were analysed using GLM with normal distribution and identity-link function (nectar volume and sugar concentration as dependent variables, and L- and S-morphs and day and night as factors) to compare the nectar traits of the two morphs between day and night. Glucose, fructose, sucrose and maltose contents in nectar were examined using GLM with normal distribution and identity-link function (sugar components as dependent variables, and L- and S-morphs as factors) to compare the sugar components between the two morphs. Data of visits were analysed using GLM with normal distribution and identity-link function (visitation rates as dependent variables, and flower morphs and visitor types as factors) to compare the visiting rates (visits/flower/hour) of all visitors between the two morphs. Pollen removal and receipt between the two morphs were compared using GLM with Poisson distribution with loglinear-link function (pollen number as dependent variable, and L- and S-

morph as factors). Seed sets of all treatments were examined with binary logistic analysis in GLM (full seed number as event variable, total ovule number as trait variable, and pollination treatments and flower morph as factors) to compare the reproductive success of six pollination treatments between the two morphs.

All data were analysed in SPSS 20.0 (IBM Inc., New York, NY) software.

Abbreviations

T. sinensis: *Tirpitzia sinensis*; L-morphs: Long-styled morphs; S-morphs: Short-styled morph; L: the length of the microcapillary tube occupied by nectar; V_{total} : the volume of one standard microcapillary; L_{total} : the length of one standard microcapillary; V: the volume of nectar; HPLC: High Performance Liquid Chromatography; GLM: generalized linear model; P/O: pollen number/ ovule number.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that there is no conflict of interest.

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Authors' contributions

XW designed the research. DH, XW, YC, MX, HT, XT carried out the field work. XW and DH analyzed the data and wrote the manuscript.

All authors contributed to writing and approved the manuscript.

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Figures

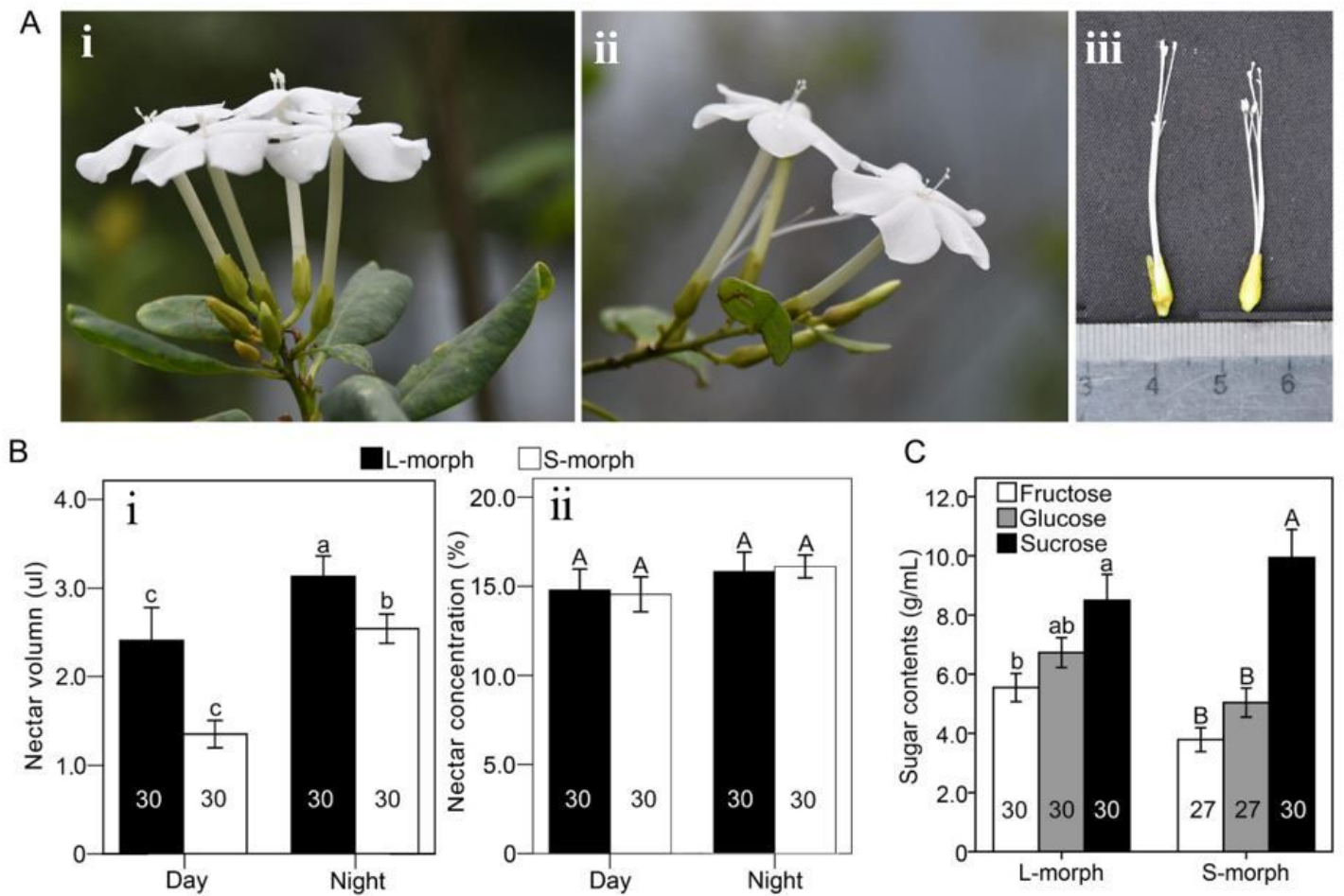


Figure 1

Photos of the short-styled (hereafter S-morph) and long-styled morphs (hereafter L-morph) of distylous *Tirpitzia sinensis*. Comparison of nectar volume, sugar concentration and composition of two morphs. A. S- (☒) and L- morphs (☒) of *Tirpitzia sinensis*. (☒) Pistil and stamen of L- and S- morphs. B. Comparison of nectar volume (☒) and nectar sugar concentration (☒) (mean \pm SE) of L- and S-morphs between day and night in *T. sinensis*. Bars that share the same letters are not significantly different between day and night phases within L- and S-morphs. C. Comparison of fructose, glucose and sucrose contents (mean \pm SE) in the L- and S-morph nectar of *T. sinensis*. Bars sharing the same letters are not significantly different between two morphs. Numbers in the bar graph represent the sample size.



Figure 2

Photos showing the main visitors of *T. sinensis* and comparison of visit rates to L- and S-morphs. A, B. Hawkmoth pollinator *Macroglossum* foraged the nectar secreted at the base of long floral tube of *T. sinensis* (Note the pollen deposited on the tongue in A, red arrow marked). C. Bumblebee (*Bombus*) always only robbed the nectar. D. Honeybees (*Apis*) mainly groomed the *T. sinensis* pollen into their corbiculae as pollen thieves (☒) when visiting the flowers.

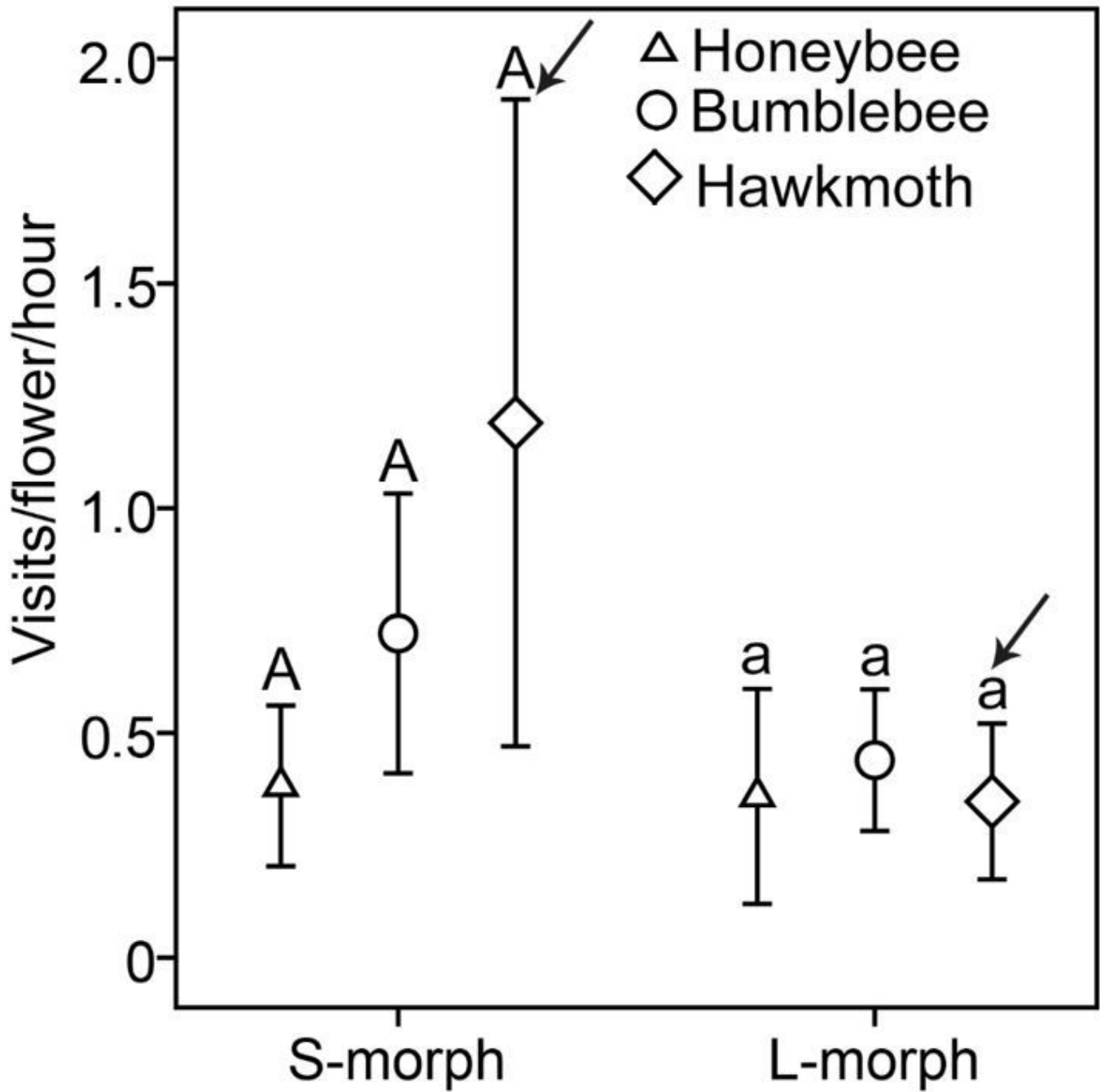


Figure 3

Comparison of visits/flower/hour between honeybee, bumblebee and hawkmoth in S- and L-morphs. Bars sharing the same letters are not significantly different in visit rates among three visitor groups in both morphs. Arrows indicated that the visit rates of hawkmoth to S-morph are significantly higher than to L-morph.

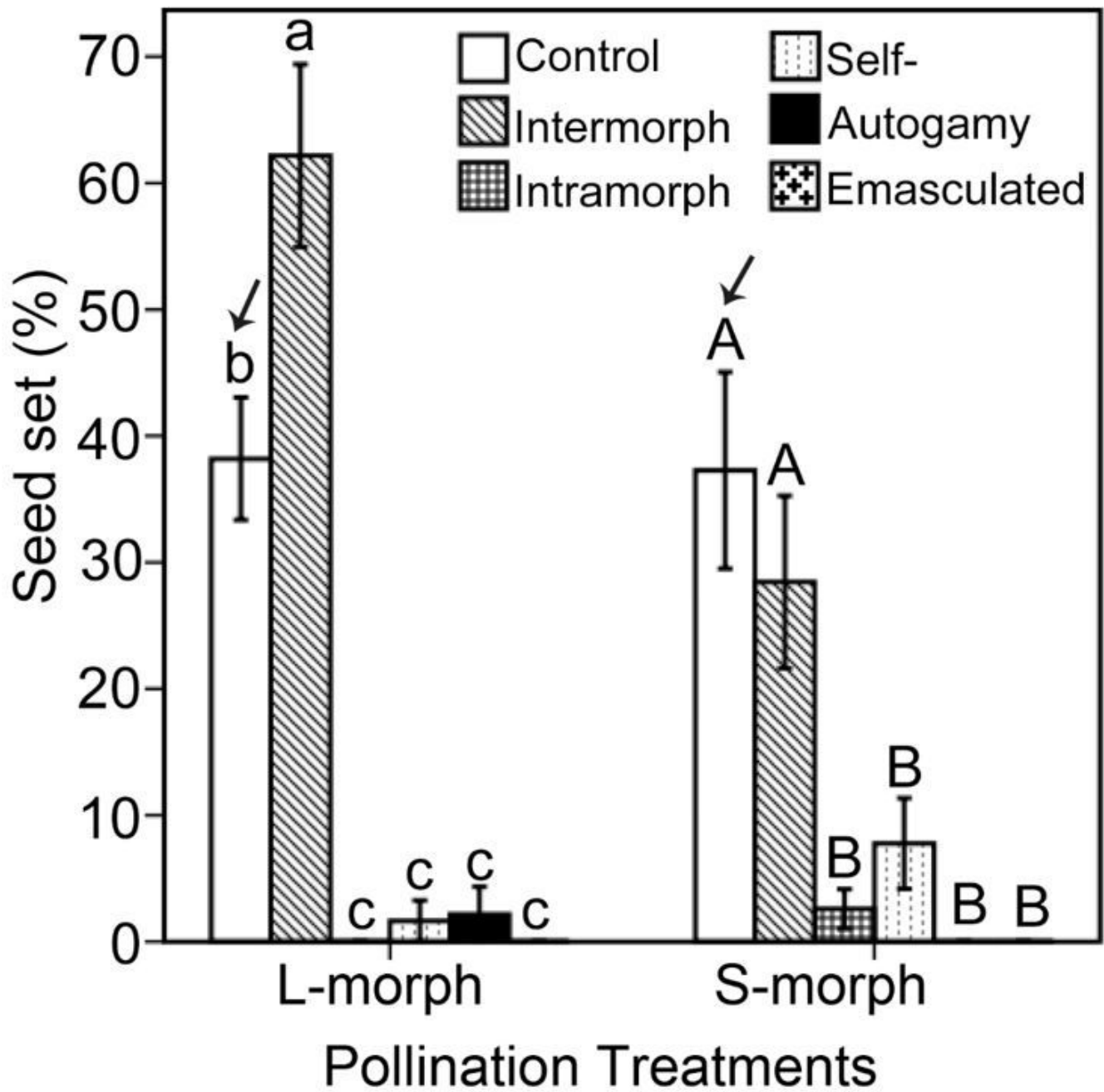


Figure 4

Comparison of seed sets under control, intermorph, intramorph, self-, autogamy and emasculated pollination treatments in L- and S-morphs. Bars sharing the same letters are not significantly different in seed set among pollination treatments. Arrows marking the seed set under control treatment between S- and L-morphs indicate no significant difference.