Journal of Tropical Ecology

www.cambridge.org/tro

Staminodes influence pollen removal and deposition rates in nectar-rewarding self-incompatible *Phanera yunnanensis* (Caesalpinioideae)

Menglin Wang[1,2,3], Shuyin Huang[1], Manru Li[1,2], Doyle McKey[4] and Ling Zhang[1,*]

[1]CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, 666303, Yunnan, China; [2]University of Chinese Academy of Sciences, 100049, Beijing, China; [3]Okinawa Institute of Science and Technology Graduate University, Tancha, Onna-son, 904-0495, Okinawa, Japan and [4]CEFE, CNRS, University of Montpellier, University Paul Valéry Montpellier 3, EPHE, IRD, 1919 route de Mende, 34293, Montpellier, France

Research Article

Cite this article: Wang M, Huang S, Li M, McKey D, and Zhang L (2019) Staminodes influence pollen removal and deposition rates in nectar-rewarding self-incompatible *Phanera yunnanensis* (Caesalpinioideae). *Journal of Tropical Ecology* **35**, 34–42. https://doi.org/10.1017/S0266467418000433

Received: 19 March 2018
Revised: 21 November 2018
Accepted: 25 November 2018

Keywords:
Phanera yunnanensis; pollen deposition; pollen removal; pollinator; staminode

Author for correspondence:
*Ling Zhang, Email: zhangl@xtbg.org.cn

Abstract

Staminodes are sterile stamens that produce no pollen, exhibit diverse structures and perform various functions. Flowers of *Phanera yunnanensis* possess three fertile stamens with large anthers and long filaments, and seven staminodes with tiny anthers and short filaments. To investigate the adaptive significance of staminodes in this species, we studied effects of staminode removal on pollen removal and deposition, flower visitation rate and fruit set in Xishuangbanna, south-western China. Four species of nectar-foraging pollinators visited flowers, mostly *Amegilla zonata* and *Apis cerana* (2.80 ± 0.15 and 1.76 ± 0.41 visits h^{-1} per flower, respectively). Staminode removal did not affect fruit set, but increased visitation by *A. cerana* by 2.6-fold, reduced visitation by *A. zonata* by 68% and increased the pollen removal rate for both pollinators (all effects were significant). Staminode removal significantly reduced pollen deposition rate for *A. zonata*, but not for *A. cerana*. These results suggest that the staminodes of *P. yunnanensis* filter which insects act as pollinators and affect pollen removal and deposition rates. By reducing pollen removal rates, staminodes may implement a pollen-dispensing schedule that spreads pollen dispersal from individual flowers over multiple pollinators. By altering pollen deposition rates, staminodes may influence reproductive fitness in other ways.

Introduction

During the evolution of the androecium in some plant lineages, some stamens have ceased to produce and present pollen. These staminodes (rudimentary and sterile stamens) have thus lost the defining function of stamens as producers of viable male gametophytes (Walker-Larsen & Harder 2000). However, like many vestigial organs, they can still play functional roles. Morphological traits of staminodes have been shown in some plants to have pollination-related functions (Hawk & Tepedino 2007, Ronse Decraene & Smets 2001). For example, the fleshy and hooded staminode in flowers of Marantaceae acts as a triggering appendage instead of producing fertile pollen grains (Ley & Claßen-Bockhoff 2012). Other studies have reported that staminodes can enhance pollination in multiple ways, such as by preventing selfing (Endress 1984), providing food for insect pollinators (Cane 1993), increasing pollinator attraction through their colours (Kaul & Koul 2012), and/or retaining staminal traits such as hairs that serve as visual cues to insect pollinators (Duffy & Johnson 2015, Lunau 2000). Sandvik & Totland (2003) found that staminodes increased pollinator visitation rate and duration in *Parnassia palustris* (Celastraceae). Flowers of *Penstemon palmeri* (Plantaginaceae) from which staminodes were removed received more visits by male *Xylocopa tabaniformis androleuca* bees, whereas frequency of visits by females of this bee was not affected; Pollen removal and deposition can also be affected by the presence or absence of staminodes in this species (Walker-Larsen & Harder 2000). The large bristle staminode of *Penstemon digitalis* appears to act as a barrier affecting bees of different body size differently, thereby influencing rates of pollen removal and pollen deposition by different bees (Dieringer & Cabrera 2002, Walker-Larsen & Harder 2001). All these studies focused mainly on the influence of staminodes on male success (Dieringer & Cabrera 2002, Walker-Larsen & Harder 2001) or on interactions with pollinators (Guimarães *et al.* 2008), whereas fewer studies have investigated staminode functions in relation to both female and male fitness.

In these previously studied cases, staminodes are quite large, robust structures, e.g. the trigger staminodes of Marantaceae and bristle staminodes of *Penstemon*. However, in many plants staminodes are small filamentous structures. We investigated the potential adaptive significance of staminodes in one such plant, *Phanera yunnanensis* (Franchet) (Wunderlin 2011) (Fabaceae,

Figure 1. Floral characters of *Phanera yunnanensis* and presentation of its pollinators. An inflorescence of *Phanera yunnanensis* at Xishuangbanna Tropical Botanical Garden (XTBG), Yunnan Province, south-western China (a). An individual flower. Three long filaments bearing fertile anthers and seven small staminodes located centrally surrounding the entrance to the nectar produced in the receptacle (b). An *Apis cerana* visiting a flower and carrying pollen at the tip of its abdomen (c). An *Amegilla zonata* visiting a flower with pollen on its abdomen (d).

Caesalpinioideae), flowers of which possess three fertile stamens and seven highly reduced filamentous staminodes. To examine the effects of staminodes on different flower visitors and on male and female reproductive functions, we studied biological and morphological characteristics and floral phenology of *P. yunnanensis*, and performed manipulative experiments removing staminodes. We specifically tested the hypotheses that staminodes influence (1) the frequency and duration of pollinator visits, and (2) pollen removal and deposition rates.

Materials and methods

Study species

Phanera yunnanensis is a perennial liana commonly found in Yunnan, Sichuan and Guizhou Provinces of China (Chen *et al.* 2010). Flowers of this species have five light purple petals (three with dark purple nectar guides), a central pistil, three large fertile stamens with longer filaments and seven staminodes with very short filaments and tiny anthers, located around the nectary found in the receptacle at the base of the flower (Figure 1a, b). Its flowers attract various visitor species, but the most common are four bee species.

Phanera (Fabaceae, Caesalpinioideae) is the largest of the several genera resulting from the reorganization of *Bauhinia* sensu lato, now considered paraphyletic and to contain polyphyletic infrageneric taxa (Sinou *et al.* 2009, Wunderlin 2010a). Molecular phylogenetic studies support the current circumscription of *Phanera* with 90–100 species all restricted to tropical Asia and Australasia (Hao *et al.* 2003, Sinou *et al.* 2009, Wunderlin 2010a). Within this cluster of lineages, ecological studies have reported diverse animal-pollination syndromes in several neotropical species of the pantropical genus *Bauhinia* sensu stricto, including bats (*B. ungulata*: Ramirez *et al.* 1984; as *B. benthamiana*), moths (*B. forficata*: Neto 2013; *B. petersiana*: Vogel 1954; as *B. macrantha*) and butterflies (*B. galpinii*: Vogel 1954). Pollination by bees and birds has been reported in two other neotropical species, *B. glabra* and *B. guianensis*, respectively (Hokche & Ramirez 1990), that are now placed in the genus *Schnella* (Wunderlin 2010b). In contrast, the floral biology and breeding systems of only three *Phanera* spp. have been studied, *P. corymbosa*, *P. glauca* and *P. championii* (Lau *et al.* 2009; all as *Bauhinia* in that study). These three species are pollinated mainly by bees and butterflies, but *P. championii* is also pollinated by wasps and flies (Lau *et al.* 2009).

Species of *Phanera* differ from almost all other members of this cluster of lineages in possessing staminodes. Almost all other species of *Bauhinia* s.l., including the sister genus of *Phanera*, *Lysiphyllum* (Wunderlin 2010a), possess 10 fertile stamens, whereas *Phanera* spp. have three (rarely two) fertile stamens and two to eight staminodes (Chen *et al.* 2010). There are no studies of the functional significance of intrafloral stamen differentiation in *Phanera*.

Study site

Our study was conducted in Xishuangbanna Tropical Botanical Garden (XTBG), Xishuangbanna, south-western China (21°56′N, 101°15′E, 580 m asl), dominated by many native forest components, including *Lagerstroemia tomentosa* (Lythraceae), *Ficus callosa* (Moraceae), *Syzygium szemaoense* (Myrtaceae) and others. XTBG is surrounded by a ~255-ha patch of relatively little-disturbed tropical rain forest. We studied two groups of *P. yunnanensis*, in two plots 1.5 km apart in the garden. The plants were introduced into the garden from nearby forest and have been

https://doi.org/10.1017/S0266467418000433

cultivated for many years in semi-garden conditions. Each group included around 10 individuals, all of which produce flowers and fruits every year.

Floral biology and phenology

We observed floral phenology of *P. yunnanensis* in two plots from July to October during 2014 and 2015. Due to the difficulty of distinguishing flowers from different individuals when several of these plants grew intermingled, we randomly selected 10 inflorescences from across the area bearing flowers in each plot, to maximize the probability that each inflorescence was from a different individual. We noted when the first flower opened and when the last one withered. At the same time, we selected 20 buds randomly from each plot, and recorded the phenology of individual flowers.

Morphological characteristics, including length and width of petals, lengths of filaments of fertile stamens and staminodes, and length of fertile anthers, were measured from about 50 flowers from each plot, using a Vernier calliper (at a scale of 0.01 mm).

We used pollen germination rate as an assay for pollen viability (Hu 1993). For this, we randomly selected 10 flowers from across the area bearing flowers in each plot, each from a different inflorescence, and bagged them using nylon mesh nets before anthesis (which generally occurs at sunrise) to prevent insect visitation. Pollen viability was tested on warm sunny days by sampling the amount of pollen grains separately from each of these 10 bagged flowers at each plot, at around 20h00 (before anthesis) and every hour from 08h00 to 11h00 on the following day (after anthesis). We measured pollen viability as per cent of pollen that germinated.

To measure stigma receptivity, we collected at least five flowers, each from a different inflorescence across the area bearing flowers in each plot, during three consecutive days under warm, sunny weather conditions at each plot every 3 h after anthesis until flower wilting. Receptivity was assayed with the method developed by Dafni & Maués (1998), which assesses stigma colour change and reaction intensity (0, 1, 2 or 3) after treatment with benzidine and hydrogen peroxide.

Preliminary observations revealed that staminodes did not produce nectar, so we sampled nectar from only the floral receptacle. We randomly selected and bagged one flower from each of 10 different inflorescences, taken across the entire flowering area in each plot, before anthesis. Volume of nectar produced by individual flowers was measured with capillary tubes (capacity = 28.3 µl) inserted into the receptacle cavity of flowers every 3 h from anthesis until flower wilting. Sugar concentration of nectar was also measured every 3 h with a refractometer (Atago Co., Ltd, range = 0–20%), using dilution samples if concentration was > 20%. Nectar collection and measuring were repeated for three consecutive days under warm, sunny weather conditions.

Mating system

The mating system of *P. yunnanensis* was unknown prior to our study. We used a series of pollination treatments to assess the capacity for autonomous autogamy and self-fertilization during the blooming peak (July–August) in 2014 and 2015. We selected about 30 individual inflorescences from across the area bearing flowers in each of the two plots, and assigned each randomly to one of eight treatments: (1) open-pollination (N, as control); (2) bagging (B, autonomous self-pollination); (3) emasculated (both stamens and staminodes removed) and bagged (EB, to test parthenogenesis); (4) emasculated and left open (RA, to test the effect of removal of both stamens and staminodes on fruit set in open-pollinated flowers); (5) staminodes removed (RSA, to test the effect of removal of staminodes on fruit set in open-pollinated flowers); (6) stamens removed (RBA, to test the effect of removal of stamens on fruit set in open-pollinated flowers); (7) hand selfing (S, to test for self-compatibility); (8) hand crossing (HC, to test for xenogamy); (9) hand geitonogamous pollinations (HG, to test for geitonogamy, i.e. fertilization resulting from transport of pollen from one inflorescence to another on the same plant). For all hand-pollination treatments, flowers were emasculated before anthesis. Fruits were collected 1 mo later and fruit set was calculated.

Further information on mating system was sought by examining pollen/ovule (P/O) ratio in flowers, which is expected to be high in obligately outcrossing plants. To determine mean P/O ratio, we collected 30 flowers from different inflorescences, three from a different plant, for each population. The three fertile anthers of each flower were fixed in 1 ml (1000 µl) 70% ethanol, then their pollen grains were squeezed out with tweezers. We then took 1 µl from the mixture and counted the number of pollen grains under a microscope (DM 2000 Leica, Germany). The numbers were then multiplied by the dilution factor of 1000 to estimate total pollen number. Ovaries of the 60 flowers from the two groups were dissected under a stereo-microscope (S8APO Leica, Germany) and the ovules counted. We then calculated the pollen/ovule ratio for each flower and the overall mean pollen/ovule ratio.

Flower visitors

Four bee species are the most frequent visitors of *P. yunnanensis* flowers. The two most common are the blue-banded bee, *Amegilla zonata* (Apidae, tribe Anthophorini, a solitary bee), and the Asian honeybee, *Apis cerana* (Apidae, a eusocial bee) (Figure 1c, d). The blue-banded bee is generally larger (average body length 12.8 mm) than the Asian honey bee (7 mm) (Jin *et al.* 2015, Zhang 2013). A third bee visitor, *Xylocopa* sp. (Apidae, tribe Xylocopini), is a large solitary bee (average body length 25 mm). *Amegilla zonata*, *Apis cerana* and *Xylocopa* sp. all forage for both nectar and pollen to feed their brood. The fourth species, *Thyreus emarginata* (Apidae, tribe Melectini; average body length = 10 mm), is a solitary cleptoparasitic cuckoo bee that forages for nectar and lays its eggs in nests of *Amegilla* spp. (Lieftinck 1968). These visitors are all native to the study region. When visiting *P. yunnanensis* flowers, their main activity is nectar collection. They are all effective pollinators, as they contact stamens or stigma during flower visits.

Manipulative experiment

To identify the functions of staminodes, we conducted two experiments during the 2014 blooming peak (July–August) with four treatments: (1) unmanipulated; (2) only staminodes removed; (3) only fertile stamens removed; and (4) all stamens and staminodes removed. Staminodes and/or stamens were removed before anthesis.

In the first experiment, we randomly selected about 10 flowers from different inflorescences (each from a different plant) for each treatment at each plot for observation of floral pollinators. Pollinator species, visitation rate and duration were recorded for each flower. *Phanera yunnanensis* produces nectar day and night. However, according to our observations, its flowers are not visited during night and most pollen grains were removed before noon on the first day of anthesis. Therefore, we recorded visitor behaviour between 08h00 and 12h00 using a video camera (Sony HDR-XR150E). The main pollinator species were identified

https://doi.org/10.1017/S0266467418000433

(viz. *Amegilla zonata* and *Apis cerana*) based on their visiting behaviour. Only visits during which bees contacted anthers, stigma or both were recorded. Pollinator visitation rates were calculated based on frequency of visitation observed during a total of 36 h spread over nine mornings during a 13-d period. The duration of each visit was measured as the time between alighting of the insect on the flower and its departure from the flower. Duration of even very short visits could be accurately measured in video recordings. We also photographed flower visitors. Voucher specimens of insects were collected and preserved in the laboratory of the Research Group 'Ecology and Evolution of Plant and Animal Interaction' at XTBG.

In the second experiment, we used the same experimental treatments to test whether the presence of staminodes and/or stamens influenced pollen removal and deposition. Staminodes and/or stamens were removed, and each bud was covered with a paper bag before anthesis. Bags were removed when observation took place, from 08h00 to 12h00. Following a single pollinator visit, anthers and stigmas were collected and fixed separately in 70% ethanol. We collected anthers and stigmas from at least 30 flowers per treatment, each from a different inflorescence. When counting pollen grains deposited on stigmas, we included both pollen grains fixed to stigmas and those washed into the ethanol. Pollen removal from visited flowers was estimated using the difference between average pollen production of flowers and residual pollen grains on the anthers of the visited flower. Number of pollen grains was estimated by crushing the fertile anthers stored in 70% ethanol before anthesis and then following the procedures described above.

Data analysis

Before conducting analyses we first checked the normality of the variables we measured, using the Shapiro–Wilk test, which is suitable for small sample sizes. If these variables were normally distributed, we used ANOVA to test whether plot had any effect on them. For the difference in filament length between staminodes and fertile stamens, we used a paired-sample *t*-test. We used two-way ANOVA to test the effects of plot and time on pollen viability, stigma receptivity, nectar volume and concentration, and then used Tukey HSD tests to analyse pairwise differences in effects for each variable. In hand-pollination experiments, because we found no fruit produced under any of the selfing treatments (B, EB, S and HG) and because data were not normally distributed, we used two-way non-parametric ANOVA (Friedman test) to analyse differences in fruit set (data on seed production per fruit were not available) by different treatments (N, RA, RSA, RBA and HC), between years and their interactions, then used LSD tests to analyse pairwise differences between treatments. We used the same method to compare total pollinator visitation rate and duration among different treatments, and differences among treatments in the frequency and duration of visits separately for each species. To compare the efficacy of pollen deposition and removal by *Amegilla zonata* and *Apis cerana*, we used two-way ANOVA to test for effects among four treatments, between two pollinators and their interactions. For numerical variables we report mean ± SE throughout. All analyses were performed using R 3.3.2.

Results

Floral biology and phenology

Flowers of *P. yunnanensis* opened around 06h00; nectar was already present when flowers opened. In each inflorescence, a mean of 1.4 ± 0.1 (n = 20, range = 0–3) flowers opened per day and individual flowers lasted 3 d. Inflorescences produced 12.4 ± 0.6 (n = 20, range = 5–17) flowers in total, with at least one open flower being present on the inflorescence during a period of 9.2 ± 0.3 d (n = 20, range = 6–11). Individual flowers produced 21.3×10^3–45.4×10^3 ($33.0 \times 10^3 \pm 0.6 \times 10^3$, n = 60) pollen grains and 5–15 (12 ± 0.4, n = 60) ovules, resulting in an average pollen/ovule ratio of 2905. Filaments of staminodes were much shorter (3.9 ± 0.002 mm, n = 94) than those of fertile stamens (20.4 ± 0.01 mm, n = 94; $t = 112$, $P < 0.001$). After anthesis, filaments of fertile stamens deflex, reaching a stable position during the first morning, whereas the style curls up, reaching its final position the following morning. None of the floral traits differed between plots (groups of plants) (Appendix 1).

This species proved to be protandrous. Some pollen grains were viable even before anthesis and pollen release began around 07h30–08h30. Pollen viability peaked (72.8% ± 4.5%, n = 10) around 09h00, then decreased until all pollen grains were removed (usually before noon; the presence or absence of pollen can be easily assessed visually). Stigma receptivity was low (1.33) at anthesis, increased quickly, reached a maximum (~2.0) from 09h00 to 15h00, then declined after that until very early the next morning. After the first night post-anthesis, stigma receptivity again increased, reaching another peak (~2.80) from 09h00 to 15h00 on the second day after anthesis. The stigma then remained receptive until the flower was shed. Pollen viability and stigma receptivity did not differ significantly between the two plots through time (Appendix 2).

Flowers secreted nectar constantly over 3 d (Figure 2); nectar quantity varied greatly among individual flowers from different plants. Mean volumes secreted were highest from 09h00 to 15h00 on the first and second days after anthesis. Nectar concentration was highest from 12h00 to 18h00 on the first and second days after anthesis, exceeding 40%. Nectar was produced during the night as well as during the day (Figure 2). Nectar concentration and volume did not differ significantly between the study plots through time (Appendix 2).

Mating system

In both 2014 and 2015, none of the selfing treatments (hand-selfing, hand-geitonogamy and bagging) resulted in any fruit set. *Phanera yunnanensis* thus appears to be self-incompatible and to require insects as pollen vectors. Among other treatments, no significant differences among treatments ($df = 4$, $F = 1.196$, $P = 0.313$) or between years ($df = 1$, $F = 2.893$, $P = 0.090$) were detected; staminode removal did not affect fruit set. The number of inflorescences in each treatment was initially about 30, but sample sizes were reduced due to inclement weather conditions and herbivores.

Flower visitation

Four nectar-foraging bee pollinators, *Amegilla zonata*, *Apis cerana*, *Thyreus emarginata* and *Xylocopa* sp., visited *P. yunnanensis* flowers. All pollinators probed for nectar immediately after landing on the centre of the flower. The visit frequencies for *Amegilla zonata* and *Apis cerana* were 2.80 ± 0.15 and 1.76 ± 0.41 visits h^{-1} per flower, respectively. Durations of their visits were 1.42 ± 0.06 and 1.61 ± 0.03 s, respectively. Frequency of *Thyreus emarginata* visits was lower (1.36 ± 0.24 visits h^{-1} per flower) and its visits were of longer duration (2.10 ± 0.18 s). Visits by *Xylocopa* sp. were both less frequent (0.31 ± 0.06 visits h^{-1} per flower) and shorter in

https://doi.org/10.1017/S0266467418000433

Table 1. Visit frequency (number of visits h^{-1} per flower) and visit duration(s) of different visitors to *Phanera yunnanensis* flowers subjected to different treatments in Xishuangbanna Tropical Botanical Garden, Yunnan Province, south-western China. Different superscript letters identify significant differences (P<0.05).

	Treatments				Test statistic	
	Unmanipulated	Removal of fertile stamens	Removal of staminodes	Removal of all stamens	F	P
Visit frequency						
Amegilla zonata	2.80 ± 0.15[a]	4.11 ± 0.26[a]	0.89 ± 0.09[b]	0.63 ± 0.04[b]	8.19	<0.001
Apis cerana	1.76 ± 0.41[a]	1.00 ± 0.08[a]	4.64 ± 0.30[b]	4.29 ± 0.31[b]	10.2	<0.001
Xylocopa sp.	0.31 ± 0.06	0.03 ± 0.01	0	0	2.11	0.122
Thyreus emarginata	1.36 ± 0.24	1.25 ± 0.22	0.07 ± 0.02	0.21 ± 0.05	1.23	0.319
Total	6.22 ± 0.24	6.39 ± 0.34	5.61 ± 0.27	5.13 ± 0.30	0.38	0.767
Visit duration						
Amegilla zonata	1.42 ± 0.06	1.42 ± 0.06	2.19 ± 0.34	1.00 ± 0.00	0.10	0.409
Apis cerana	1.61 ± 0.03	1.61 ± 0.03	1.87 ± 0.06	1.54 ± 0.05	1.20	0.328
Xylocopa sp.	0.69 ± 0.09	0.69 ± 0.09	0	0	2.74	0.063
Thyreus emarginata	2.10 ± 0.18	2.10 ± 0.18	2.00 ± 0.46	0.67 ± 0.16	0.69	0.569
Total	1.58 ± 0.03	1.54 ± 0.05	1.97 ± 0.08	1.49 ± 0.04	1.74	0.183
N	9	9	7	6		

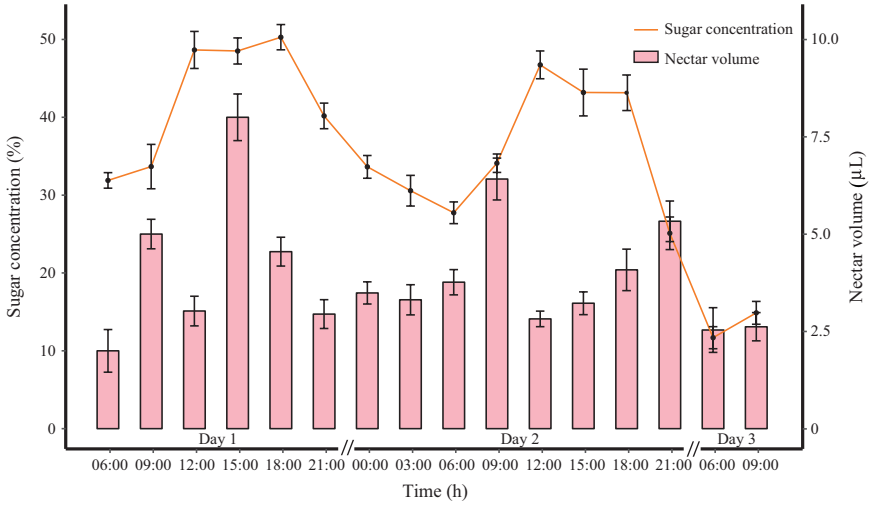

Figure 2. Mean (± SE) sugar concentration (%) and nectar volume (μl) accumulated since the last observation in *Phanera yunnanensis* flowers during the first two days of flowering (n = 20) at Xishuangbanna Tropical Botanical Garden (XTBG), Yunnan Province, south-western China. Days of flowering were separated by breaks, Day 1: the day when flower opens; Day 2: the second day after anthesis; Day 3: the third day after anthesis.

duration (0.69 ± 0.09 s) than for any of the other three bee species. Flower visit frequency and duration by the two last species did not differ significantly among the four treatments (Table 1). We thus excluded them from further analyses of results of the pollen removal and deposition experiments.

Overall frequency and duration of visits by all pollinators combined did not differ significantly among the four treatments, but behaviour of both *Amegilla zonata* and *Apis cerana* during visits did differ among treatments (Table 1). Removal of staminodes, and of both staminodes and stamens, led to decreased visit frequency by *Amegilla zonata* relative to the other two treatments (unmanipulated or flowers from which only stamens were removed). In contrast, flowers from which staminodes, or both stamens and staminodes, had been removed received more visits by *Apis cerana* than did unmanipulated flowers or flowers from which only stamens had been removed. *Xylocopa* sp. did not visit flowers from which staminodes had been removed, regardless of whether fertile stamens were present. Visit duration for these four species did not differ significantly among the four treatments (Table 1).

Pollen deposition and removal

For pollen grain deposition, there were significant differences among flowers under different treatments ($F = 25.3$, $P < 0.01$), but not between the two principal pollinators *Amegilla zonata* and *Apis cerana* ($F = 2.35$, $P = 0.13$), and there were no interaction effects between treatments and pollinators ($F = 1.76$, $P = 0.16$) (Appendix 3). Unmanipulated flowers received significantly more pollen than all the different kinds of manipulated flowers after a single visit by *Amegilla zonata* ($F = 21.5$, $P < 0.001$) (Figure 3a). In contrast to *Amegilla zonata*, pollen deposition by *Apis cerana* did not differ significantly between unmanipulated flowers and flowers with staminodes or stamens plus staminodes removed. Only flowers without fertile stamens (but with staminodes) received significantly

https://doi.org/10.1017/S0266467418000433

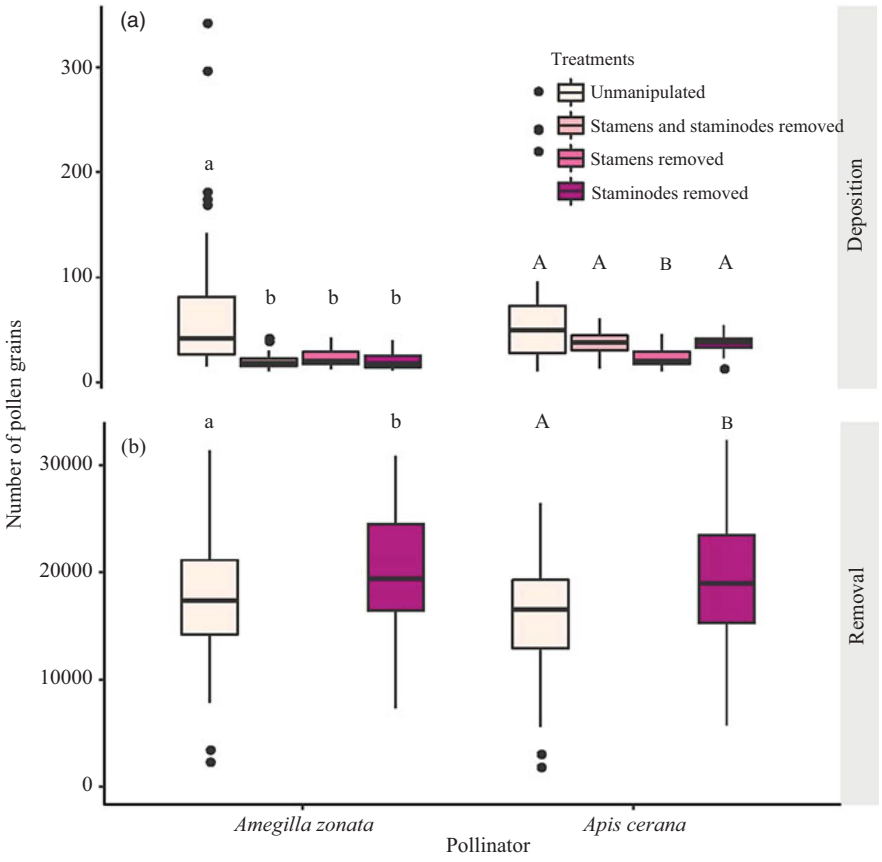

Figure 3. Mean (± SE, n = 30) amount of pollen grains deposited on stigmas (a) and pollen removed (b) of *Phanera yunnanensis* during first visits by *Amegilla zonata* and *Apis cerana* to unmanipulated flowers and to flowers from which staminodes had been removed at Xishuangbanna Tropical Botanical Garden (XTBG), Yunnan Province, south-western China. Different letters indicate significant differences (P<0.05).

less pollen during *Apis cerana* visits ($F = 18.0$, $P < 0.001$) (Figure 3a). Pollen removal also showed the same pattern: different treatments had a significant effect on pollen removal ($F = 12.0$, $P < 0.01$), but there was no significant effect of different pollinators ($F = 1.02$, $P = 0.31$), and no interaction effects between treatments and pollinators ($F = 0.632$, $P = 0.61$) (Appendix 4). Pollen removal by both *Amegilla zonata* and *Apis cerana* increased significantly after staminode removal compared with unmanipulated flowers (for *Amegilla zonata*, $t = -2.22$, $P = 0.029$; for *Apis cerana*, $t = -1.00$, $P < 0.001$) (Figure 3b).

Discussion

Floral biology

The results of hand-pollination experiments and the high pollen/ovule ratio (typical of primarily outcrossed plants; Cruden 1977) both show that *Phanera yunnanensis* is self-incompatible and requires for its reproduction insects that transport pollen between individual plants. Previously studied species of *Phanera* are also self-incompatible (Lau *et al.* 2009). Several floral characteristics of *P. yunnanensis* facilitate attraction of nectar-foraging pollinators. First, extended floral display likely enhances pollinator attraction, potentially increasing reproductive success (Jin *et al.* 2015). Second, prolonged receptivity of the stigma provides more time for the stigma to receive pollen, enhancing the opportunity for female reproductive success. Whereas all pollen grains were removed during the first two mornings after anthesis, the stigma remained receptive for 3 d until the flower was shed. Flowers of *P. yunnanensis* in effect turn to female phase after all pollen has been removed. Similar results have been found in *Jatropha curcas* (Euphorbiaceae) (Luo *et al.* 2007). Such temporal separation of sex roles, or dichogamy, reduces interference between female and male organs within the same flower, promoting outcross-pollen transfer (Barrett 2002). Third, the movement herkogamy (spatial separation of sexual organs within flowers) of anthers and stigma that we observed in *P. yunnanensis* should reduce within-flower self-pollination, as has been observed in *Ourisia macrocarpa* (Scrophulariaceae) (Schlessman 1986) and *Eremurus himalaicus* (Liliaceae) (Verma *et al.* 2004).

Nectar is the main reward to pollinators of *P. yunnanensis*. In other plants, high sugar concentration is known to increase the duration of pollinator visits (Gleiser *et al.* 2014). Although nectar volume in *P. yunnanensis* is lower than that observed in other species of *Bauhinia* s.l., the concentration of its nectar is much higher (Lau *et al.* 2009, Neto 2013). In our study, two peaks of nectar production were observed, during the mornings of the first and second days after anthesis, which also coincided with peaks in anther dehiscence and pollinator visits. Although nectar was produced continuously, volume and concentration of nectar were higher in the day than at night, coinciding with mostly diurnal visitor activity. Higher nectar concentration during the day could also result from greater evaporation. However, if evaporation was the whole story, nectar volume should also be lower in the day than at night, but that is not the case in *P. yunnanensis*. The continuous production of nectar in *P. yunnanensis* could maintain the attractiveness of flowers to pollinators.

Flower visitation

Phanera yunnanensis flowers attracted four species of nectar-foraging bees, two of which were the main pollinators, *Amegilla*

https://doi.org/10.1017/S0266467418000433

zonata and *Apis cerana*. The open corolla of *P. yunnanensis* flowers allows easy access to multiple flower visitors. Staminodes have been reported to perform various functions in flowering plants, sometimes different from those assured by fertile stamens, including providing nectar and/or enhancing visual attraction to increase frequency of pollinator visits (Cronquist 1981, Endress 1984). In *P. yunnanensis* staminodes are neither conspicuous in colour nor nectar-rewarding, and the overall frequency and duration of visits, for all pollinators combined, to flowers with staminodes and those from which staminodes had been removed did not exhibit significant differences. However, staminodes of *P. yunnanensis* have different effects on different pollinators. Removal of staminodes decreased the frequency of visits by *Amegilla zonata*, but increased the frequency of visits by *Apis cerana*, and had no effect on the two other species of pollinators (Table 1).

Staminodes have been shown to perform different pollination-related functions in several plant species (Dieringer & Cabrera 2002, Sandvik & Totland 2003, Walker-Larsen & Harder 2000, 2001). For example, Dieringer & Cabrera (2002) found that of eight bee species attracted to the flowers of *Penstemon digitalis* (Plantaginaceae), only the medium-sized bee *Anthophora terminalis* (Apidae) avoided visiting flowers from which the staminode had been removed, while both larger and smaller bee species exhibited no preference between unmanipulated flowers and those with the staminode removed. That study proposed that the large bristle-like staminode constituted a barrier to some pollinators. Walker-Larsen & Harder (2001) found that function of the staminode in *Penstemon palmeri* was related to its location in relation to corolla shape. In *P. yunnanensis*, none of the mechanisms by which staminodes have been proposed to affect pollinator visits in other plants appears to apply. Further research should explore the mechanisms acting in this species.

Pollen removal and deposition by different pollinators

Our results show that removal of staminodes increased the amount of pollen removed per visit for both *Amegilla zonata* and *Apis cerana*. By limiting the amount of pollen removed per visit, staminodes enable *P. yunnanensis* to increase the number of visits until all pollen is removed and thereby maximize the opportunities for pollen to be carried by different pollinator individuals. In *Penstemon digitalis*, Dieringer & Cabrera (2002) reported that removal of a large bristle staminode resulted in fewer pollen grains being deposited on stigmas and less pollen being removed from anthers by both large and small bees, but the contrary was true for medium-sized bees. That study suggested that the bristle staminode functions as a barrier for many pollinators, preventing excessive removal of pollen grains (Dieringer & Cabrera 2002).

Whatever the mechanism by which staminodes influence duration of visits by different pollinators of *P. yunnanensis*, our results suggest that their effects alter pollen packaging (Harder & Thomson 1989), spreading pollen dispersal across a larger number of pollinator visits and thereby perhaps increasing male fitness. In addition, we observed that after removal of staminodes, pollen deposition on stigmas by one of the two main pollinators (*Amegilla zonata*) decreased significantly, while no change was detected for the other (*Apis cerana*). For *Apis cerana*, pollen deposition decreased only when it visited flowers from which fertile stamens had been removed. The presence of staminodes influences pollinator composition and visitation frequency, which together control rates of pollen removal and deposition. Regardless of what the mechanisms are, the overall effect of staminodes is to reduce the amount of pollen removed per visit, so that pollen is dispensed over a larger number of pollinator visits.

Conclusions

In this study, staminode removal significantly increased the frequency of visits by *Apis cerana*, but reduced the frequency of visits by *Amegilla zonata*, while the pollen removal rate was increased for both pollinator species. Staminode removal had different effects on pollen deposition by these two species. In unmanipulated flowers, pollen deposition did not differ between the two species. Whereas removal of staminodes had no effect on pollen deposition by *A. cerana*, staminode removal led to a dramatic decrease in pollen deposition by *Amegilla zonata*. Thus, staminodes of *P. yunnanensis* might alter the way in which amounts of pollen are dispensed across visits. By influencing pollinator composition and visitation, staminodes can influence pollen removal and deposition rates, promoting an optimal pollen-dispensing schedule for presenting pollen to their pollinators. Further studies are needed to investigate the mechanisms by which staminodes differentially affect pollen deposition and removal by different pollinators of *P. yunnanensis* and to determine whether such differential effects of staminodes are observed in other species in the genus. Like *P. yunnanensis*, each of the three other species of *Phanera* whose pollination ecology has been investigated is also visited by diverse insects (Lau *et al.* 2009), suggesting considerable scope for such differential effects.

Author ORCIDs. Menglin Wang https://orcid.org/0000-0003-2206-9503.

Acknowledgements. We thank Mr Qiaoshun Li and Changzong Zhu of Xishuangbanna Tropical Botanical Garden for their assistance in field experiments, Dr Jin Zhao for assistance with some data analysis and Professors Richard Corlett, Xiaodong Yang and Jin Chen, also of Xishuangbanna Tropical Botanical Garden, for their constructive suggestions and comments. We thank the reviewers for comments that greatly improved the manuscript. We also thank Dr Ravi K. Chaturvedi for English language editing.

Financial support. This work was supported by the National Natural Science Foundation of China (31670393) awarded to L. Zhang.

Literature cited

Barrett SCH (2002) Sexual interference of the floral kind. *Heredity* **88**, 154–159.

Cane JH (1993) Reproductive role of sterile pollen in cryptically dioecious species of flowering plants. *Current Science* **65**, 223–225.

Chen DCH, Zhang DX, Larsen K and Larsen SS (2010) *Bauhinia. Flora of China* **10**, 6–21.

Cronquist A (1981) *An Integrated System of Classification of Flowering Plants*. New York: Columbia University Press, 1262 pp.

Cruden RWR (1977) Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. *Evolution* **31**, 32–46.

Dafni A and Maués MM (1998) A rapid and simple procedure to determine stigma receptivity. *Sexual Plant Reproduction* **11**, 177–180.

Dieringer G and Cabrera LR (2002) The interaction between pollinator size and the bristle staminode of *Penstemon digitalis* (Scrophulariaceae). *American Journal of Botany* **89**, 991–997.

Duffy KJ and Johnson SD (2015) Staminal hairs enhance fecundity in the pollen-rewarding self-incompatible lily *Bulbine abyssinica*. *Botanical Journal of the Linnean Society* **177**, 481–490.

Endress PK (1984) The role of inner staminodes in the floral display of some relic Magnoliales. *Plant Systematics and Evolution* **146**, 269–282.

Gleiser G, Internicol AAI, Austerlitz F and Bernasconi G (2014) Stabilizing selection on nectar concentration in wild *Petunia axillaris*, as revealed by genetic analysis of pollen dispersal. *Evolutionary Ecology* **28**, 869–884.

Guimarães E, Di Stasi LC and Mainoni-Rodella RDCS (2008) Pollination biology of *Jacaranda oxyphylla* with an emphasis on staminode function. *Annals of Botany* **102**, 699–711.

https://doi.org/10.1017/S0266467418000433

Hao G, Zhang DX, Zhang MY, Guo LX and Li SJ (2003) Phylogenetics of *Bauhinia* subgenus *Phanera* (Leguminosae: Caesalpinioideae) based on ITS sequences of nuclear ribosomal DNA. *Botanical Bulletin of Academia Sinica* **44**, 223–228.

Harder LD and Thomson JD (1989) Evolutionary options for maximizing pollen dispersal of animal-pollinated plants. *American Naturalist* **133**, 323–344.

Hawk JL and Tepedino VJ (2007) The effect of staminode removal on female reproductive success in a Wyoming population of the endangered Blowout Penstemon, *Penstemon haydenii* (Scrophulariaceae). *Madroño* **54**, 22–26.

Hokche O and Ramirez N (1990) Pollination ecology of seven species of *Bauhinia* L. (Leguminosae: Caesalpinioideae). *Annals of the Missouri Botanical Garden* **77**, 559–572.

Hu SY (1993) Experimental methods in plant embryology (i) determination of pollen viability. *Chinese Bulletin of Botany* **10**, 60–62.

Jin H, Jiao GJ and Chen G (2015) Observation on flowering process, floral morphology and flower visiting insects of *Melastoma candidum* f. *albiflorum*. *Journal of Plant Resources and Environment* **24**, 73–79.

Kaul V and Koul AK (2012) Staminal variation and its possible significance in *Commelina benghalensis* L. and *Commelina caroliniana* Walter. *Current Science* **103**, 419–426.

Lau CPY, Saunders RMK and Ramsden L (2009) Floral biology, breeding systems and population genetic structure of three climbing *Bauhinia* species (Leguminosae: Caesalpinioideae) in Hong Kong, China. *Journal of Tropical Ecology* **25**, 147–159.

Ley AC and Claßen-Bockhoff R (2012) Floral synorganization and its influence on mechanical isolation and autogamy in Marantaceae. *Botanical Journal of the Linnean Society* **168**, 300–322.

Lieftinck MA (1968) A review of Old World species of *Thyreus* Panzer (= *Crocisa* Jurine) (Hym., Apoidea, Anthophoridae) Part 4. Palearctic species. *Zoologische Verhandelingen* **98**, 1–139.

Lunau K (2000) The ecology and evolution of visual pollen signals. *Plant Systematics and Evolution* **222**, 89–111.

Luo C, Li K, Chen Y, Sun Y and Yang W (2007) Pollen viability, stigma receptivity and reproductive features of *Jatropha curcas* L. (Euphorbiaceae). *Acta Botanica Boreali-Occidentalia Sinica* **27**, 1994–2001. (In Chinese with English abstract.)

Neto HFP (2013) Floral biology and breeding system of *Bauhinia forficata* (Leguminosae: Caesalpinioideae), a moth-pollinated tree in southeastern Brazil. *Brazilian Journal of Botany* **36**, 55–64.

Ramirez N, Sobrevil AC, Enbech NXD and Ruiz-Zapata T (1984) Floral biology and breeding system of *Bauhinia benthamiana* Taub.(Leguminosae), a bat-pollinated tree in Venezuelan "llanos". *American Journal of Botany* **71**, 273–280.

Ronse Decraene LP and Smets EF (2001) Staminodes: their morphological and evolutionary significance. *Botanical Review* **67**, 351–402.

Sandvik SM and Totland Ø (2003) Quantitative importance of staminodes for female reproductive success in *Parnassia palustris* under contrasting environmental conditions. *Canadian Journal of Botany* **81**, 49–56.

Schlessman MA (1986) Floral protogyny, self-compatibility and the pollination of *Ourisia macrocarpa* (Scrophulariaceae). *New Zealand Journal of Botany* **24**, 651–656.

Sinou C, Forest F, Lewis GP and Bruneau A (2009) The genus *Bauhinia* s.l. (Leguminosae): a phylogeny based on the plastid Trn L–Trn F region. *Botany* **87**, 947–960.

Verma S, Magotra R and Koul AK (2004) Stylar movement avoids self pollination and promotes cross pollination in *Eremurus himalaicus*. *Current Science* **87**, 872–873.

Vogel S (1954) Blütenbiologische Typen als Elemente der Sippengliederung. *Botanical Studies* **1**, 1–338.

Walker-Larsen J and Harder LD (2000) The evolution of staminodes in angiosperms: patterns of stamen reduction, loss, and functional re-invention. *American Journal of Botany* **87**, 1367–1384.

Walker-Larsen J and Harder LD (2001) Vestigial organs as opportunities for functional innovation: the example of the *Penstemon* staminode. *Evolution* **55**, 477–487.

Wunderlin RP (2010a) Reorganization of the Cercideae (Fabaceae: Caesalpinioideae). *Phytoneuron* **48**, 1–5.

Wunderlin RP (2010b) New combinations in *Schnella* (Fabaceae: Caesalpiniodeae: Cercideae). *Phytoneuron* **49**, 1–5.

Wunderlin RP (2011) New combination in *Phanera* (Fabaceae). *Phytoneuron* **19**, 1–2.

Zhang Z (2013) *Reproductive Ecology of* Phalaenopsis pulcherrima *(Orchidaceae), a Species Endemic to East Asia*. PhD dissertation. Hai Nan University, Haikou, China. (In Chinese with English abstract.)

Appendix 1

Comparison of the floral traits of *Phanera yunnanensis* in two plots, using two-sample *t*-tests in Xishuangbanna Tropical Botanical Garden (XTBG), Yunnan Province, south-western China

Floral traits	Mean ± SE		Test statistic		
	plot 1	plot 2	*t* test	df	P
No. of flowers per inflorescence	11.9 ± 0.3	13.0 ± 0.1	−0.919	18	0.376
Duration per inflorescence (d)	9.0 ± 0.2	9.4 ± 0.1	−0.647	18	0.528
Pollen grains (10^3)	32.6 ± 0.2	33.5 ± 0.1	−0.714	58	0.478
Ovules	11.6 ± 0.08	12.0 ± 0.05	−0.882	58	0.382
P/O ratio (10^3)	3.0 ± 0.04	2.8 ± 0.01	0.779	58	0.441
Petal length (mm)	24.3 ± 0.02	24.2 ± 0.03	0.350	92	0.728
Petal width (mm)	10.7 ± 0.01	10.6 ± 0.02	0.260	92	0.796
Fertile anther length (mm)	4.11 ± 0.00	4.07 ± 0.01	0.775	92	0.441
Fertile anther filament length (mm)	20.5 ± 0.02	20.4 ± 0.04	0.376	92	0.708
Staminode filament length (mm)	3.88 ± 0.01	3.94 ± 0.01	-0.881	92	0.381

Appendix 2

Tests for differences in floral traits of *Phanera yunnanensis* over the duration of flowering and between the two plots, using two-way ANOVA, in XTBG, Yunnan Province, south-western China

Floral traits		F	P
Stigma receptivity	Time	3.49	0.009
	Plot	3.22	0.091
	Time × Plot	1.17	0.303
Pollen viability	Time	1.47	0.358
	Plot	7.61	0.051
	Time × Plot	1.26	0.3
Nectar volume	Time	0.91	0.569
	Plot	3.64	0.077
	Time × Plot	4.85	0
Sugar concentration	Time	6.47	0.001
	Plot	1.72	0.212
	Time × Plot	6.05	0

https://doi.org/10.1017/S0266467418000433

Appendix 3

Deposition of pollen of *Phanera yunnanensis* by *Amegilla zonata* and *Apis cerana* under different treatments (unmanipulated, fertile stamens removed, staminodes removed and all stamens removed) and their interactions, in XTBG, Yunnan Province, south-western China, compared using two-way ANOVA

Predictor variable		F	P
Treatment	Unmanipulated	25.3	<0.01
	Fertile stamens removed		
	Staminodes removed		
	All stamens removed		
Pollinator	*Amegilla zonata*	2.35	0.13
	Apis cerana		
Treatment × Pollinator		1.76	0.16

Appendix 4

Removal of pollen of *Phanera yunnanensis* by *Amegilla zonata* and *Apis cerana* under different treatments (unmanipulated and staminodes removed) and their interactions, compared using two-way ANOVA, in XTBG, Yunnan Province, south-western China

Predictor variable		F	P
Treatment	Unmanipulated	12.0	<0.01
	Staminodes removed		
Pollinator	*Amegilla zonata*	1.02	0.31
	Apis cerana		
Treatment × Pollinator		0.26	0.61

https://doi.org/10.1017/S0266467418000433