PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Floral divergence, pollinator partitioning and the spatiotemporal pattern of plant–pollinator interactions in three sympatric *Adenophora* species

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Abstract Floral divergence among congeners may relate to differential utilization of pollinators and contribute to reducing overlap in pollination niches. To investigate whether and how floral differences are associated with differential utilization of pollinators in three sympatric *Adenophora* species, we analyzed floral traits and evaluated the contribution of different visitors to pollination. We compared visitation rates of different pollinator categories in different years and sites. A suite of floral traits differed among the three *Adenophora* species, suggesting adaptation to diurnal versus nocturnal pollination and an intermediate condition. However, many visitor species were shared among the three plant species, suggesting that floral traits did not rigorously filter visitors. Effective pollinators were large bees and moths. The importance of large bees as pollinators decreased whereas that of moths increased along the gradient from typically bee-pollinated to moth-pollinated flowers. The intermediate species (*A. khasiana*) differed substantially from the other two species in pollinator species but not in pollinator categories. The principal pollinator category of each species was constant across years and sites except in the intermediate species where it differed between two sites. Overall, the three sympatric species of *Adenophora* partition pollinators by floral divergence and the principal pollinators coincide with the predictions based on floral syndromes.

Introduction

Related species in sympatry may evolve discrete pollination syndromes, thereby partitioning pollinators, reducing overlap in pollination niches (Kay and Schemske 2005; Wolfe and Sowell 2006; Martin and Willis 2007; Dell'Olivo et al. 2011), and minimizing the detrimental effects of interspecific pollen transfer (see Armbruster et al. 1994; Muchhala and Potts 2007). Floral divergence associated with transition in pollinator categories has been documented in numerous taxa (reviewed in Johnson and Steiner 2000; Fenster et al. 2004; Goldblatt and Manning 2006; Thomson and Wilson 2008; Johnson 2010; van der Niet and Johnson 2012). Partitioning pollinators does not necessarily entail completely different pollination syndromes, involving whole suites of floral traits. For example, subtle morphological modification may result in different pollen placement sites on the same pollinator (Armbruster et al. 1994; Stone et al. 1998; Pauw 2006; Muchhala and Potts 2007; Botes et al. 2008; Schiestl and Schlüter 2009; Huang and Shi 2013). In such cases, related species in sympatry may use the same pollinators with some limited overlap in pollination niches. Thus, the partitioning of pollinators seems to be readily achieved in specialized plant–pollinator interactions.

While comparative pollination studies continue to highlight the striking association between floral syndromes and pollination systems (Fenster et al. 2004; van der Niet and Johnson 2012), large-scale and community-wide surveys generally reveal that a given plant species commonly

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attracts a range of animal species (Waser et al. 1996; Olesen and Jordano 2002; Ollerton et al. 2009), casting doubt on the existence of specialized plant–pollinator interactions in community contexts (Ollerton et al. 2007; Kessler and Baldwin 2011). In lineages with nectar and pollen well exposed and available to various visitors, the overlap of pollinators may be substantial among sympatric related species. Many floral visitors do not seem to match expectations based on floral traits, and the unexpected visitors can sometimes transfer pollen (Ollerton et al. 2007). Moreover, "there may be a widespread unconscious bias to ignore visitors that seem 'improper' under the paradigm of pollination syndromes" (Waser et al. 1996). Species may experience substantial interspecific pollen transfer if various pollinators are shared in generalized pollination systems. However, not all visitors contribute equally to the pollination of a plant (Ollerton 1996; Fenster et al. 2004). For example, among diverse visitors foraging on *Clerodendrum trichotomum* flowers, only a swallowtail and a carpenter bee were effective pollinators (Sakamoto et al. 2012). To clarify pollen transfer dynamics among species with generalized flowers, experimental designs should be adopted that quantify the roles of different visitors in pollination.

The extent and pattern of pollinator partitioning among related species in sympatry may vary with years and sites if relative abundance of different floral visitors varies in time and space (Herrera 1988, 1989; Schemske and Horvitz 1989; Ollerton 1996; Brunet 2009; Artz et al. 2010; Fang and Huang 2012). For example, Artz et al. (2010) observed substantial spatiotemporal variation in pollinator composition and abundance on two subspecies of *Oenothera cespitosa* in which the floral syndrome predicts moth pollination. They found that medium-sized hawkmoths and large matinal bees were the principal effective pollinators, but their relative importance varied yearly and spatially. Therefore, an investigation of variation in the composition, abundance, and effectiveness as pollinators of the flower visitor spectrum in different community contexts is essential for an understanding of the relationship between sympatric congeners and pollinators.

To examine how co-existing closely related species with relatively generalized flowers reduce potential reproductive interference, we explored the reproductive ecology of three species of alpine ladybells (*Adenophora*) in southwest China. Flowers of *Adenophora* are presumed to be mainly pollinated by bumblebees (Roquet et al. 2008). However, a shift from bee pollination to moth pollination can be assumed based on floral syndromes (Susanne Renner, personal communication). We predict that the blue flowers are pollinated by bumblebees and the white flowers are pollinated by moths in the study species. The species with variable floral color may attract both bumblebees and moths. On the other hand, other foraging insects may also be attracted

because pollen and nectar are not fully concealed. Our objectives were to investigate whether the three sympatric *Adenophora* species partition pollinators as predicted by floral syndromes, and whether the plant–pollinator interactions vary among years and sites.

Materials and methods

Study species and site

The ladybells *Adenophora jasionifolia*, *A. khasiana*, and *A. capillaris* (Campanulaceae) are perennial herbs endemic to southwest China. They grow in alpine meadows or the edges of forests. The three species produce stems each with a terminal inflorescence, which has 1–8 blue flowers and no branches in *A. jasionifolia*, 15–60 whitish to blue flowers and often a few small branches in *A. khasiana*, and large racemose cymes with about 20–100 white flowers in *A. capillaris*. Anthesis lasts approximately 3.5 days in *A. jasionifolia*, 5 days in *A. khasiana*, and 6 days in *A. capillaris*. Each corolla tube of the ladybells has five lobes. The inflated bases of filaments (we call them nectar covers) cover the secretory floral disc and the nectar. Pollen grains shed from the anthers before anthesis are held among pollen-collecting hairs on the dorsal surfaces of stigmatic lobes in the male phase. This type of secondary pollen presentation with protandry is typical in the Campanulaceae (Erbar and Leins 1995; Muchhala 2006).

The three species were studied in sympatry at a field station in Shangri-La Alpine Botanical Garden (SABG), Yunnan Province, southwest China (27°54′23″N, 99°38′30″E; 3,200 m altitude) where *A. jasionifolia* was evenly distributed on the slopes and *A. khasiana* and *A. capillaris* grew in sparse large patches. The three species bloomed approximately from late July to early September at the field station where we quantified floral traits and conducted pollination experiments. Plant–pollinator interactions were also investigated at two other sites. At each of these, two of the studied plant species occurred. *Adenophora khasiana* and *A. capillaris* co-existed at Cangshan Mountain, Dali, Yunnan Province (25°44′26″N, 100°07′39″E; 2,700 m altitude), while *A. capillaris* (28°21′23″N, 98°57′40″E; 3,550 m altitude) and *A. jasionifolia* (28°21′18″N, 98°57′39″E; 3,700 m altitude) grew in allopatry at Shengping, Deqin, Yunnan Province.

Floral traits

To compare the floral morphology of the three species, we measured corolla length, corolla orifice diameter, and stigma exsertion in 2009 in SABG. We also recorded the timing of pollen and nectar presentation. Since the timing

Table 1 Comparison of floral traits in three *Adenophora* species

	A. jasionifolia	*A. khasiana*	*A. capillaris*	Difference among species
Attraction				
Color	Blue	Whitish to blue	White	
Odor	Absent	Absent or light	Present	
Morphology				
Corolla lobes	Not reflexed	Not reflexed	Reflexed	
Pedicel texture	Thick, stiff	Thick, stiff	Slender, soft	
Corolla tube	Campanulate, pendent	Funnel-form, pendent	Urceolate, nodding	
Stigma exsertion (mm)	3.74 ± 0.34b	4.78 ± 0.40b	13.35 ± 0.39a	$F = 88.07$
Corolla length (mm)	18.44 ± 0.37b	20.59 ± 0.36a	13.90 ± 0.19c	$H = 82.10$
Orifice diameter (mm)	29.19 ± 0.80a	24.65 ± 0.41b	4.95 ± 0.13c	$H = 194.19$
Nectar cover	Tight	Loose	Loose	
Nectar				
Concentration (%)	42.9 ± 0.019a (day)	39.8 ± 0.010a (day)	19.2 ± 0.006b (night)	$H = 63.63$
		17.9 ± 0.004b (night)		
Secretion (day), (µl)	2.56 ± 0.193a	0.46 ± 0.040b	–	$Z = -7.44$
Secretion (night), (µl)	–	0.60 ± 0.030b	1.71 ± 0.084a	$Z = -7.32$

Means ($\pm$SE) are shown for quantitative traits ($n = 40$ for all measurements; except for nectar concentration, for which $n = 21$). Statistical tests for among species differences in traits are shown in the last columns

Values with different letters are significantly different at $P = 0.05$ among the three species

of pollen presentation can be associated with specific pollination agents (Stone et al. 1998; Thomson et al. 2000), we recorded whether anthesis of a flower had begun at half-hour intervals. To characterize the tempo of nectar secretion, in 2010 the nectar covers were removed and the nectar was absorbed into filter paper strips from each flower; a small cotton ball filled the corolla tube to prevent visitation. A thread was fastened on the corolla tube to fix the cotton ball in *A. jasionifolia* and *A. khasiana* flowers. The nectar was left to accumulate from 0700 to 1700 hours (diurnal secretion) and from 1700 to 0700 hours the next morning (nocturnal secretion). The volume of accumulated nectar and the sugar concentration were measured with capillary tubes and a pocket refractometer. One flower was measured per plant, but the nectar from more than one flower in a plant was used if the amount of nectar in one flower was too small to measure concentration. We chose male-stage flowers for morphological measurements and female-stage flowers for nectar measurements because the female stage lasted longer and the three species were all protandrous. Color, odor, corolla, and corolla lobe shape and pedicle texture were recorded (see Table 1). Data on style exsertion were normally distributed and were analyzed with one-way ANOVAs followed by Tukey tests. The other data in this study were analyzed with Mann–Whitney *U* tests or Kruskal–Wallis tests followed by Mann–Whitney *U* tests due to non-normality and/or non-homogeneity of variances. All analyses were performed in SPSS v.16.0.

Overlap in flowering time

To document flowering time, we monitored flowering individuals of each species within six 4-m² quadrats in the garden once every 3 days to record the number of blooming flowers until all the flowers on these plants wilted. We recorded 1,512, 2,662, and 3,166 blooming flowers in *A. jasionifolia*, *A. khasiana*, and *A. capillaris*, respectively, through the observation period in 2010, and 1,860, 3,050, and 3,529 blooming flowers, respectively, in 2011. To depict the overlap of flowering time among the three species, we plotted their flowering curves. For each species, the number of open flowers recorded per count was divided by the total number of open flowers recorded throughout the flowering season so that each point in the curve designates the percentage of the total number of flowers recorded (see Martin and Willis 2007).

Flower visitors

To examine whether visitors discriminate among the three species, we observed flower visitors in summer 2009, 2010, and 2011 in Shangri-La. To detect geographical variation in visitor assemblages and visitation rates, we observed visitors in Dali and Deqin in 2011. To obtain one sample of visitation rate, the visits by all visitors of different categories were recorded during one observation period (half an hour) in sunny weather. Visitors were subdivided into three different categories in terms of behavior on flowers

and activity time, including large bees, moths, and small bees and flies. We shifted to another patch before proceeding to the next observation period. Only one or two samples of visitation data were obtained within any single day. The visitation rate per flower per hour was estimated as the number of visits per observation period multiplied by two and divided by the number of observed flowers. Nocturnal observation began at 2010 hours; moths did not emerge earlier. A small flashlight covered with thick red plastic film was used during nocturnal observation. Our observation periods were scattered from August 5 to 20 for *A. jasionifolia* and *A. capillaris*, and from August 10 to September 5 for *A. khasiana*. During each observation period, we observed about 20, 30, and 100 flowers of *A. jasionifolia*, *A. khasiana*, and *A. capillaris*, respectively. These flower numbers were appropriate given that both the visitation rate and spatial density of flowers differed among species.

Pollination experiments

To examine the relative importance of diurnal and nocturnal pollinators, we conducted five treatments in 2009 in SABG. The treatments started when the floral buds were about to open and finished after anthesis. (1) Diurnal pollination: flowers were exposed after sunrise and bagged after sunset. (2) Nocturnal pollination: flowers were exposed after sunset and bagged after sunrise. (3) Control: flowers were always exposed. Two additional treatments were conducted to examine the potential of autogamy and seed set after cross-pollination. (4) Autogamy: flowers were always bagged. (5) Hand pollination: the flowers were bagged and hand-pollinated with cross pollen from another plant more than 10 m away. In each treated plant, at least the first four treatments were conducted, and in some plants, the last treatment was also conducted. Each of the five treatments was conducted on one flower per plant. In 2010, we repeated the above treatments except autogamy. The nylon nets for pollinator exclusion were fixed on bamboo poles thrust into the earth close to the stems, to avoid the risk of the nets pressing on flowers. More than 3 weeks after the flowers had wilted, we harvested the fruits and counted seeds and ovules per fruit, and calculated seed set per fruit.

Pollen deposition and pollinator importance

To estimate the contributions of different diurnal visitor categories to pollination, we calculated the number of pollen grains deposited per visit and pollinator importance (visitation rate × number of pollen grains deposited) (Reynolds et al. 2009). The flowers were bagged before the female phase. Once the stigmas matured, the flowers were exposed to visitors. The stigmas were collected after one insect visit and pollen grains deposited on each stigma

were counted under a stereomicroscope. Conspecific pollen could not be distinguished from heterospecific pollen but we could at least estimate the quantity of pollen deposited by different visitor categories.

Results

Floral traits

Trait analysis revealed considerable floral difference in traits of attraction (color and odor), morphology, nectar, and the timing of anthesis between three *Adenophora* species (Table 1; Fig. 1). Compared to *A. capillaris* which had white fragrant flowers, dilute nectar, and loose nectar covers, *A. jasionifolia* had dark blue scentless flowers, concentrated nectar, and nectar covers that seemed difficult to penetrate through, given that small bees had to bite holes in the nectar covers to get nectar. While *A. jasionifolia* secreted nectar during the day, *A. capillaris* began to secrete at night. Compared to the former two species, *A. khasiana* had intermediate floral traits. Its flowers did not emit perceptible fragrance, floral color varied among individuals from blue to whitish, and it secreted nectar throughout the day, but nectar concentration differed between day and night (Table 1). The three species bloomed and started to present pollen at different times of day. Most flowers of *A. jasionifolia* opened around midday, while all flowers of *A. capillaris* opened at dusk. Flowers of *A. khasiana* were also inclined to open late within a day but not as regularly as those of *A. capillaris* did (Fig. 1).
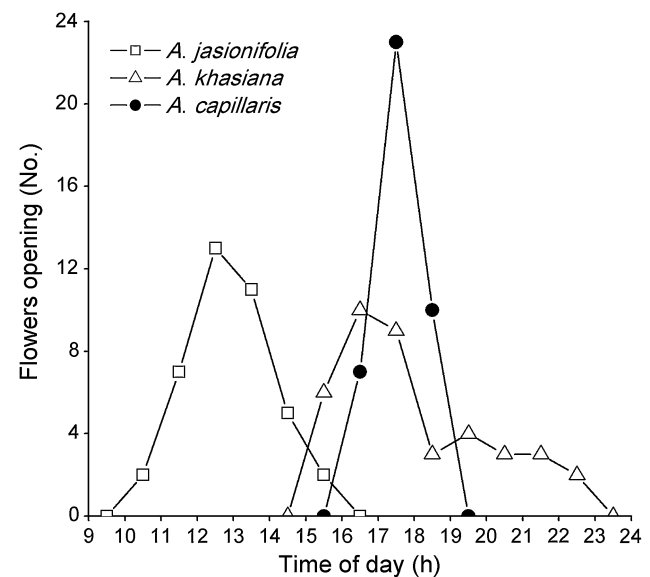

Fig. 1 Timing of anthesis in three *Adenophora* species. Data were determined from observations of 40 plants of each species in 2009
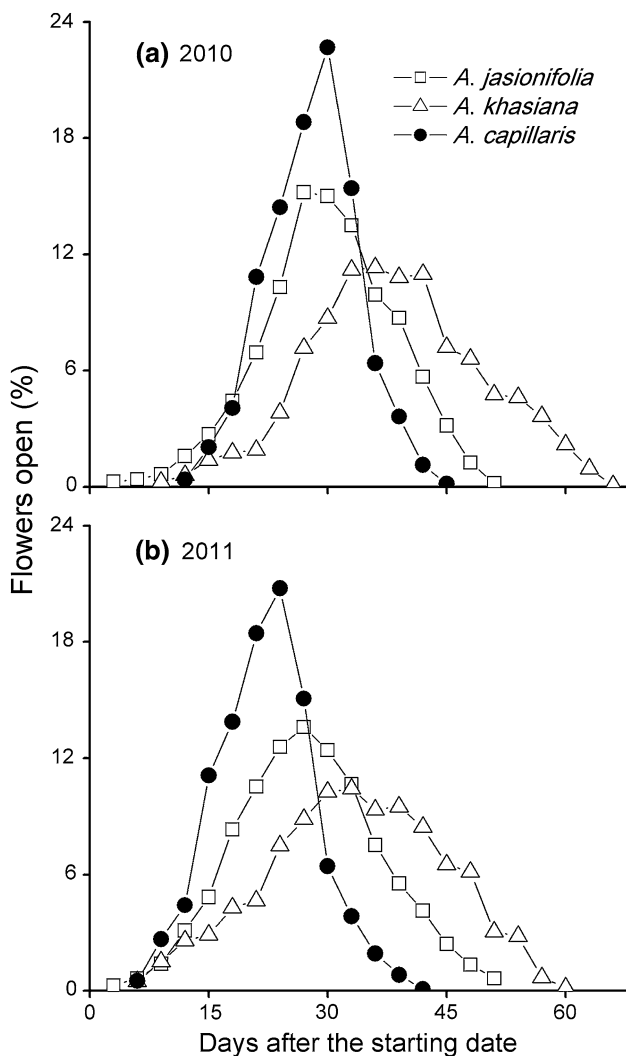
Fig. 2 Flowering phenology of three *Adenophora* species in **a** 2010 and **b** 2011 at Shangri-La Alpine Botanical Garden. In each year, the proportion of open flowers of a given day was calculated for each species from six 4-m² quadrats

Flowering phenology among the three species overlapped to some extent: *A. jasionifolia* and *A. capillaris* reached flowering peak synchronously (Fig. 2); *A. khasiana* and *A. capillaris* started to bloom at the same time but the overlap of their flowering phenology was less; and while *A. khasiana* had a long flowering season, *A. capillaris* seemed to burst into bloom and to cease flowering abruptly.

Visitor observations

A variety of insects was recorded visiting flowers of three *Adenophora* species in SABG, including bees, flies, moths, beetles, earwigs, and mites (Table 2), confirming our prediction that the relatively open flowers would attract a broad assemblage of floral visitors. Given that bumblebees

and honeybees behaved in similar ways and had larger bodies, we use the term "large bees" here to contrast them with other visitors. The other bees and flies contributed little to pollination and were combined in the same group ("small bees and flies") in the statistical analysis.

Large bees

Large bees were the most frequent pollinators in *A. jasionifolia* and *A. khasiana* (Fig. 3a, b, d). *Adenophora jasionifolia* was visited by bumblebees and not by honeybees while honeybees were dominant bees visiting *A. khasiana* (90.8 % in 2009, 76.5 % in 2010, and 78.5 % in 2011). Visitation rates of large bees differed among three *Adenophora* species in 2009, 2010, and 2011 ($H = 23.10$, $H = 30.24$, and $H = 34.93$, all $P < 0.0001$), respectively (Fig. 4). The visitation rate of large bees was higher in *A. jasionifolia* than in *A. khasiana* ($P = 0.004$) in 2010 but not in other years ($P > 0.5$), and was lowest in *A. capillaris* in all 3 years ($P < 0.0003$). Among the 3 years, the visitation rate in *A. jasionifolia* was highest in 2010, and in *A. khasiana* was highest in 2011 ($P < 0.02$). Large bees contacted the stigmatic lobes or pollen with their ventral abdomens. In *A. khasiana* and *A. capillaris*, large bees sometimes took an inappropriate posture and did not touch the pollen or stigmas so precisely as in *A. jasionifolia* (Fig. 3d).

Small bees and flies

The bees and flies rarely touched the receptive stigmatic surfaces, and they sometimes acted as pollen thieves (Fig. 3c, i) and delivered little pollen (see "Pollen deposition and pollinator importance"). Their visitation rate differed among three *Adenophora* species in 2009 ($H = 34.62$, $P < 0.0001$), 2010 ($H = 33.17$, $P < 0.0001$), and 2011 ($H = 9.16$, $P = 0.01$) (Fig. 4). The visitation rate was the highest in *A. jasionifolia* ($P < 0.005$) and lowest in *A. capillaris* ($P < 0.03$) in the 3 years of study, except that there was no significant difference between *A. jasionifolia* and *A. khasiana* ($P = 0.43$) in 2011.

Moths

In the 3 years of study, we did not observe any moths visiting *A. jasionifolia*. Furthermore, moth scales were not observed on stigmatic lobes in *A. jasionifolia* but were common in the other two species (Fig. 3e, h). Five moth species, *Thysanoplusia intermixta*, *Autographa excelsa*, *Autographa nigrisigna*, *Autoculeora locuples*, and *Panchrysia tibetensis*, dominated the visitations (86.8 % in 2009, 68.7 % in 2010, 79.8 % in 2011) in *A. capillaris* but they played a minor role in *A. khasiana* (0 % in 2009, 11.4 % in 2010, 27.3 % in 2011). The visitation rate of moths was

Table 2 Insect species observed visiting flowers of three *Adenophora* species in Shangri-La during summer 2009–2011

Visitors	*A. jasionifolia*	*A. khasiana*	*A. capillaries*
Bees	*Bombus richardsi*	*Apis cerana*	*Bombus richardsi*
	Lasioglossum sp. 1	*Bombus richardsi*	*Bombus festivus*
	Lasioglossum sp. 2	*Lasioglossum* sp. 1	*Apis cerana*
	Unidentified solitary bee	*Lasioglossum* sp. 2	*Lasioglossum* sp. 2
Moths		Noctuidae	
		Mythimna separata	*Autographa excelsa*
		Autographa excelsa	*Autographa nigrisigna*
		Thysanoplusia intermixta	*Autoculeora locuples*
		Albocosta ellapsa	*Thysanoplusia intermixta*
		Albocosta musiva	*Panchrysia tibetensis*
		Agrotis trifurca	*Euxoa intolerabilis*
		Platoplusia tancrei	*Mythimna separata*
		Agrotis sp.	*Dictyestra reticulata*
		Mythimna sp.	*Albocosta ellapsa*
		Dictyestra sp.	*Trichoridia canosparsa*
		Xestia sp.	*Actebia* sp.
			Hadena sp.
			Agrotis sp.
			Heliophobus sp.
			Unidentified sp. 1
			Unidentified sp. 2
		Others	
		Arctiidae sp.	Geometridae sp.
		Pyralididae sp.	Pyralidae sp.
Flies	*Syrphus ribesii*	*Syrphus ribesii*	*Episyrphus balteatus*
	Asarkina sp.	*Asarkina* sp.	*Asarkina* sp.
	Sphaerophoria sp.		*Musca* sp.
Beetles	Chrysomeloidea sp.	Chrysomeloidea sp.	
Earwigs	*Forcipula* sp.	*Forcipula* sp.	*Forcipula* sp.
Mites	Tetranychidae sp.		Tetranychidae sp.

significantly higher in *A. capillaris* than in *A. khasiana* in 2010 ($Z = -2.75$, $P = 0.005$), but not in 2009 ($Z = -1.24$, $P = 0.22$) and 2011 ($Z = -0.81$, $P = 0.43$) (Fig. 4). In *A. capillaris*, the visitation rate was higher in 2010 than in 2009 ($P = 0.02$). The moths alighted on flowers by catching hold of the small reflexed corolla lobes with the two forelegs. As the moth hung onto the corolla, its proboscis was extended to probe inside the flowers and sometimes the head entered the floral tube. In the course of feeding, the wings kept vibrating, the body shaking slightly with the flower. The moth's abdomen could thus contract the pollen or stigmatic lobes (Fig. 3h).

Beetles, earwigs and mites

Beetles and mites visited flowers to feed on pollen grains. Earwigs took both pollen and nectar. These insects stayed on flowers for so long that we could not calculate their visitation rate. We rarely observed them moving among flowers or touching stigmas (Fig. 3f). We thus defined them as pollen or nectar thieves.

Observations at the other study sites

Although the visitation rate and pollinator species differed among study sites, the principal pollinator categories of *A. jasionifolia* and *A. capillaris* were constant (Fig. 4). The visitation rate of bumblebees (*Bombus convexus*) to *A. jasionifolia* was far lower in Deqin than that of *B. richardsi* in Shangri-La ($Z = -3.57$, $P = 0.0002$). Moths did not visit *A. jasionifolia* in either site. Moths and very few bumblebees were observed to visit *A. capillaris* in Deqin and Dali. The visitation rate of moths was lower in Dali than in Shangri-La (marginal significance, $Z = -1.95$, $P = 0.051$)
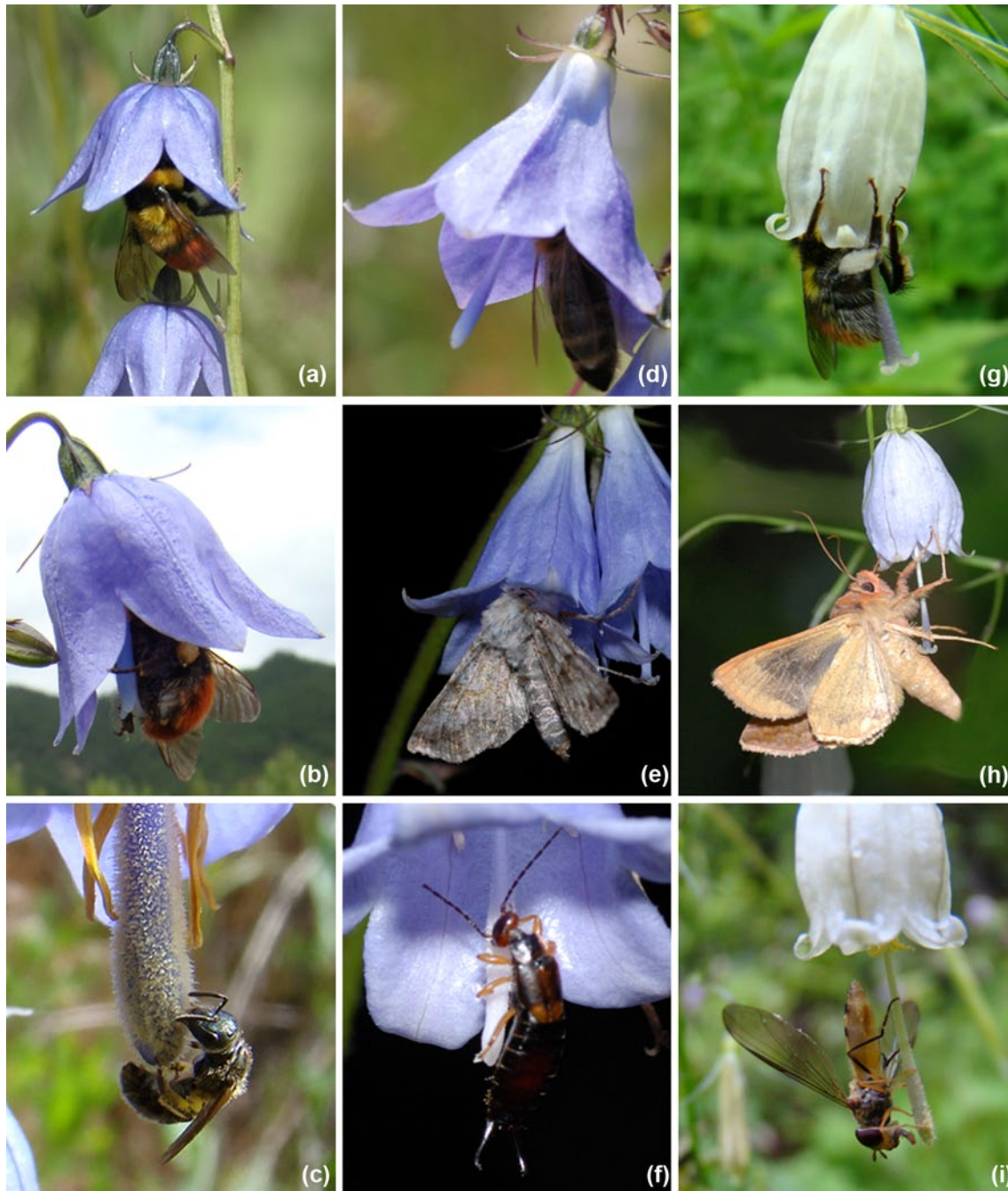
Fig. 3 How different pollinators enter flowers to collect nectar or pollen from the three *Adenophora* species. **a–c** *A. jasionifolia*. **d–f** *A. khasiana*. **g–i** *A. capillaris*. **a, b, g** *Bombus richardsi* sucking nectar. **c** *Lasioglossum* sp. 2 collecting pollen. **d** *Apis cerana* sucking nectar. **e** *Platoplusia tancrei* sucking nectar. **f** *Forcipula* sp. consuming pollen. **h** *Autographa excelsa* sucking nectar. **i** *Episyrphus balteatus* consuming pollen

and Deqin ($Z = -3.16$, $P = 0.001$). In Dali *Thysanoplusia intermixta*, *Autographa excelsa* and *Autoculeora locuples* dominated the visits to *A. capillaris* (77.5 %), but common visitors in Shangri-La (*Panchrysia tibetensis* and *Autographa nigrisigna*) were not observed there. In Deqin, the moths *Autographa excelsa* and *Autoculeora locuples*

accounted for only 13.3 % of the visits. The other moths were *Albocosta*, *Polia*, and *Sideridis* species, none of which were found in Shangri-La and Dali.

The principal pollinators of *A. khasiana* switched from large bees in Shangri-La to moths in Dali. In Dali, we only observed four visits by a bumblebee (*Bombus funerarius*)

Fig. 4 Visitation rates of three pollinator categories to different *Adenophora* species at **a–c** Shangri-La, in different years, and at **d** Deqin and **e** Dali in 2011. *Bars* mean ± SE; the numbers of observation periods are shown above each *bar*
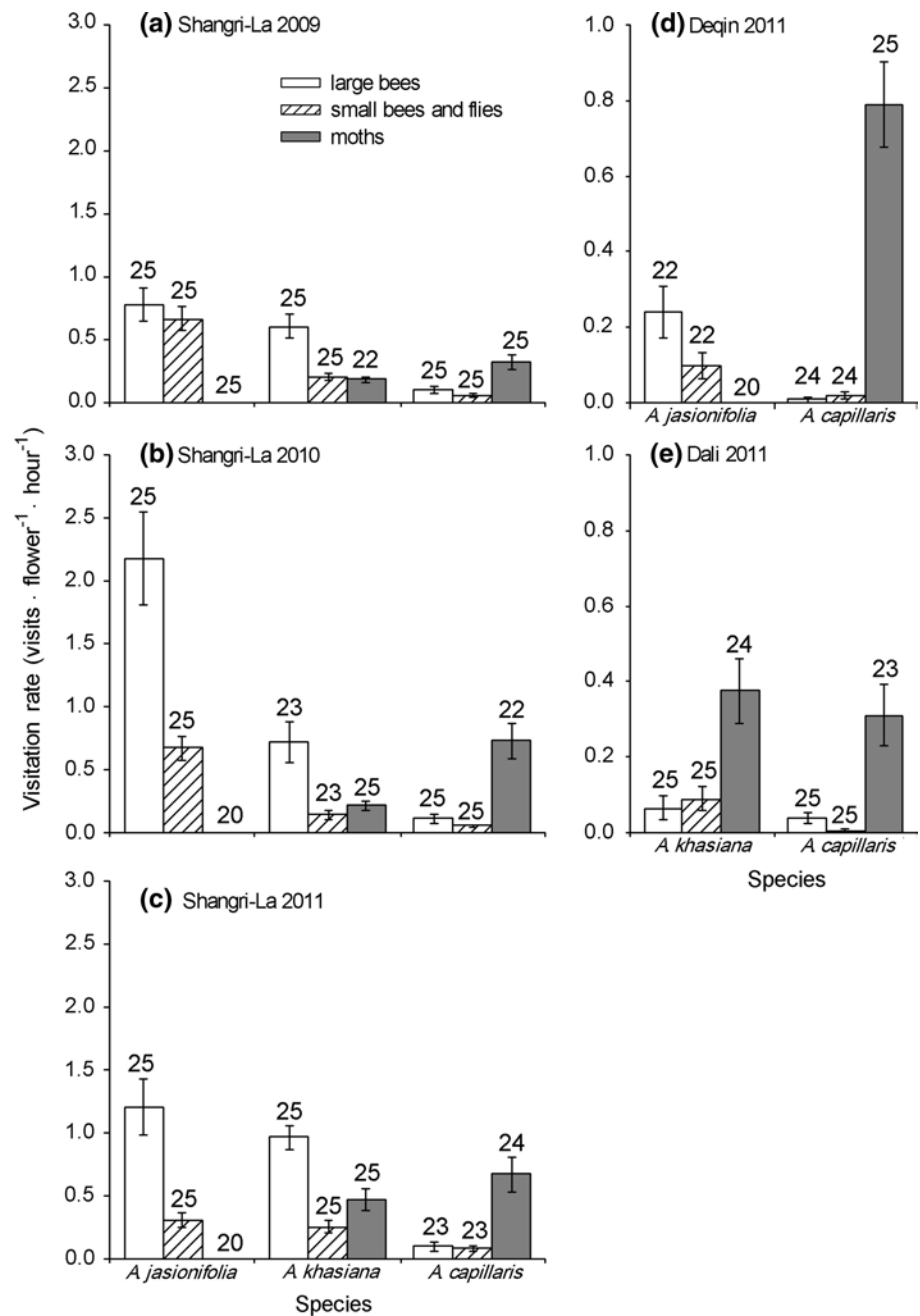
and no honeybees visited flowers during the observation periods despite their notable abundance on sympatric flowers. In contrast to Shangri-La, the moths *Thysanoplusia intermixta*, *Autographa excelsa*, and *Autoculeora locuples* accounted for 84.2 % of the total moth visits to *A. khasiana* in Dali. The flowers emitted a faint fragrance in Dali but no perceptible odor in Shangri-La.

Pollination experiments

Seed sets were significantly different among the five pollination treatments in each of the three *Adenophora* species

in 2009 (*A. jasionifolia*: $H = 142.25$, $n = 40$ in diurnal pollination, 40 in nocturnal pollination, 40 in control, 40 in autogamy, 37 in hand pollination; *A. khasiana*: $H = 111.01$, $n = 40, 40, 40, 40, 31$; *A. capillaris*: $H = 109.66$, $n = 34, 34, 34, 34, 31$, all $P < 0.0001$) and 2010 ($H = 193.71$, $n = 60, 60, 60, 60, 40$; $H = 231.39$, $n = 96, 96, 96, 40, 78$; $F = 228.38$, $n = 96, 96, 96, 34, 78$, all $P < 0.0001$) (Fig. 5). The three species set very few seeds by autogamy (seed set <3 %). In *A. jasionifolia*, seed set was not significantly enhanced by hand pollination compared to open-pollinated controls ($P > 0.5$), suggesting no pollen limitation. Seed set under night pollination (2.1 %) was not significantly
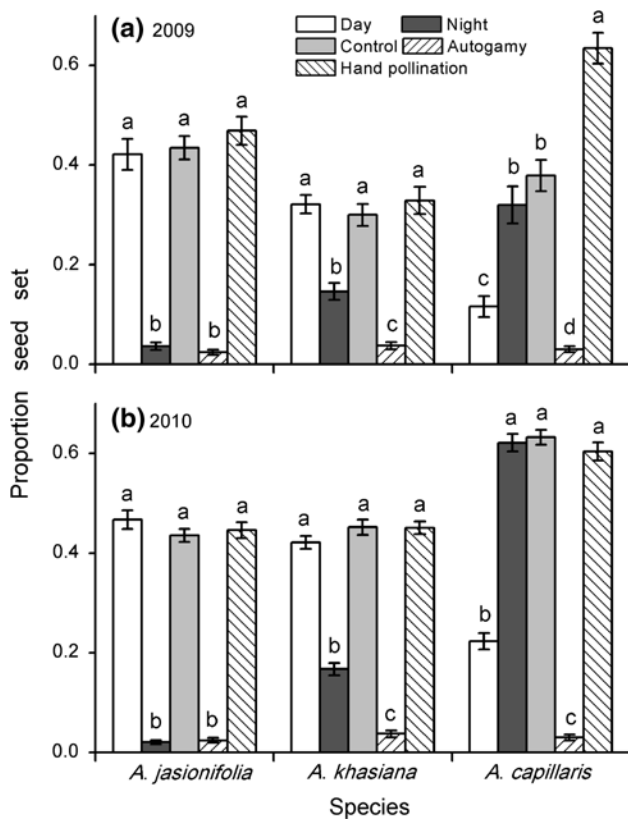
Fig. 5 Proportion seed set per flowers under different pollination treatments in the three *Adenophora* species in **a** 2009 and **b** 2010. *Bars* mean ± SE; *different letters* above *bars* indicate significant differences between treatments within species

different from that of the autogamy treatment ($P > 0.2$), suggesting that pollination in *A. jasionifolia* depended exclusively on diurnal pollinators, consistent with pollinator observations (moths were not observed). In *A. jasionifolia*, bumblebees were effective and reliable pollinators in that pollen limitation was absent even in 2009 when bumblebees were relatively scarce. In *A. khasiana*, too, no pollen limitation was observed in either year ($P > 0.4$). In *A. khasiana*, both the day and night pollination treatments yielded higher seed set than the autogamy treatment ($P < 0.0001$), suggesting that both diurnal and nocturnal

pollinators contributed to pollination. However, diurnal visitors were more important than nocturnal visitors as pollinators ($P < 0.0001$) in terms of seed set (Fig. 5). Diurnal pollinators (compared to hand pollination, $P > 0.06$) rather than nocturnal pollinators ($P < 0.0001$) could achieve adequate pollination for the species. In *A. capillaris*, pollen limitation was observed in 2009 ($P < 0.0001$) but not in 2010 ($P = 0.144$). Both day and night pollination yielded higher seed set than did autogamy ($P < 0.0001$), suggesting that both diurnal and nocturnal pollinators contributed to pollination. In contrast to *A. khasiana*, in *A. capillaris* seed set was significantly lower under day pollination than under night pollination ($P < 0.0001$), indicating that moths rather than bees were major pollinators in this white-flowered species.

Pollinator importance and pollen deposition

Large bees deposited significantly more pollen grains ($P < 0.0001$) per visit than did small bees and flies in each of the three species (Table 3). Combining pollen deposition and visitation rate, the pollination importance of large bees was more than 50 times that of small bees and flies, indicating that large bees contributed most to diurnal pollination and that solitary bees and flies played only a negligible role as pollinators in these *Adenophora* species.

Discussion

We found that the pattern of plant–pollinator interactions in three *Adenophora* sympatric species across years and populations was consistent with predictions based on floral syndromes. According to our pollinator observations and pollination treatments, the principal pollinators were bumblebees, honeybees, and moths in *A. jasionifolia*, *A. khasiana*, and *A. capillaris*, respectively, in Shangri-La in the 3-year study. Flower preference by bees and moths was associated with the gradient of floral variation from typical bee flowers to typical moth flowers. In line with this, the categories of principal pollinators did not vary among years or sites in *A. jasionifolia* and *A. capillaris* despite

Table 3 Pollen deposition on stigmas of virgin flowers of three *Adenophora* species after one visit by the two categories of pollinators and pollinator importance

	Pollen deposition		Pollinator importance	
	Large bees	Small bees and flies	Large bees	Small bees and flies
A. jasionifolia	296.0 ± 42.7 (50)	3.4 ± 2.2 (47)	356.4 ± 51.4	1.4 ± 1.3
A. khasiana	115.7 ± 25.5 (35)	1.2 ± 0.9 (26)	112.4 ± 20.9	0.5 ± 0.4
A. capillaris	107.8 ± 27.7 (27)	2.7 ± 1.7 (25)	10.4 ± 2.7	0.2 ± 0.1

Pollinator importance is calculated following Reynolds et al. (2009) including both pollen deposition and visitation rate. Values are mean ± SE (*n*)

considerable spatiotemporal variation in visitation rates and pollinator species. *Adenophora khasiana* with intermediate floral traits was pollinated principally by large bees or moths in different populations and partitioned pollinator species with the other two sympatric congeners in Shangri-La. As predicted, each *Adenophora* species attracted a broad assemblage of floral visitors, but only large bees and/or moths were effective pollinators.

Floral difference and pollinator preference

Utilization of various pollinators by plant species with overlapping flowering time can be associated with divergence in floral traits. Differences in visitation rates of the different visitor categories in the three co-flowering sympatric *Adenophora* species indicated that visitors were sensitive to the variation in floral traits. For example, the timing of pollen and nectar presentation in *A. capillaris* corresponded with the activity time of moths; the white corolla and fragrance may help moths to discover the flowers in the dark. The nectar sugar concentration in *A. capillaris* was far from the optimal concentration for bees (see Kingsolver and Daniel 1995) but similar to other moth-pollinated flowers (e.g., Nilsson et al. 1990; Makholela and Manning 2006; Reynolds et al. 2009). *Adenophora khasiana* and *A. capillaris* have no mechanical barriers against large bees but the former species was mainly visited by honeybees and the latter was occasionally visited by any large bees, indicating that pollinator preference related to floral difference in attraction and reward.

Both the attraction of large bees (visitation rate) and the dependence on large bees for seed set decreased from phenotypically bee-adapted (*A. jasionifolia*) to typically moth-adapted flowers (*A. capillaris*) (Figs. 4, 5), while the attraction and dependence on moths varied in the opposite way. The floral syndrome and pollinator categories of *A. khasiana* both seem to be intermediate between those of *A. jasionifolia* and *A. capillaris*. Wilson et al. (2006) suggested that plants with intermediate traits may use both categories of pollinators and exhibit "despecialization" in pollination, compared to species with definite floral syndromes and mainly employing one category of pollinators. It is noteworthy that despecialization in *A. khasiana* does not lead to a pollination system in which any visitors become pollinators (similar cases are described in Macior 1986; Manning and Goldblatt 2005; Pérez et al. 2006; Alcantara and Lohmann 2010).

Flowers may be visited and pollinated by various animals not predicted by pollination syndromes (Waser et al. 1996; Ollerton et al. 2007). Highly specialized floral phenotypes would filter visitors other than those with proper characters (Armbruster 1984, 2012; Buchmann 1987; Johnson 1994; Schiestl et al. 1999; Martins and Johnson 2007;

Hentrich et al. 2010). Nevertheless, flowers with nectar and/or pollen readily available such as *Adenophora* species here may often be visited by various visitors, as suggested by Waser et al. (1996). We have demonstrated that the *Adenophora* flowers attracted broad assemblages of visitors, but visitors other than large bees and moths acted mainly as pollen and/or nectar thieves. Compared with large bees, the small bees and flies could deliver very few pollen grains in all three *Adenophora* species, evidently due to their unsuitable body sizes. Beetles, mites, and earwigs did worse in that they rarely moved between flowers and also could hardly contact stigmas. In addition, the visitation rate of low-efficiency small bees and flies tended to decrease when the floral syndrome approached moth pollination from *A. jasionifolia* to *A. capillaris* (Fig. 4). Low-efficiency floral visitors can exert selection on plants as antagonists and may affect the evolution of floral traits (Lau and Galloway 2004; Hargreaves et al. 2010). Thus, the change in attraction of the low-efficiency visitors may play an additional role in the evolutionary transition between pollination syndromes.

Pollinator partitioning

Closely related species can partition pollinators through subtle floral modifications (e.g., Armbruster et al. 1989; Kay 2006; Pauw 2006; Muchhala and Potts 2007; Botes et al. 2008; Waelti et al. 2008; Huang and Shi 2013) or complete divergence in pollination syndromes (Fulton and Hodges 1999; Chari and Wilson 2001; Ramsey et al. 2003; Kay and Schemske 2005; Wilson et al. 2006; Wolfe and Sowell 2006). The *Adenophora* species whose stigmas picked up pollen from the ventral abdomens of large bees and moths (Fig. 3) were unlikely to use different sites on the same pollinator to carry pollen. Instead, the timing of pollen presentation and pollinator activity differed among the three species. For example, white-flowered *A. capillaris* was generally visited first by moths at night so that pollen grains were transferred first by moths among conspecific plants before bees emerged the next day. The earlier visits by moths reduced reproductive interference by limiting pollen available to bees which might cause interspecific pollination. Therefore, floral divergence associated with pollinator behavior may reduce pollen loss caused by interspecific visitation (Muchhala and Thomson 2012). In addition, seed sets under the natural pollination treatments were not lower than those in the hand pollination treatments in the three species except in *A. capillaris* in 2009, suggesting that the effect of interspecific pollination on seed production could be minimal. The moth visitation rate was significantly lower in 2009 than in 2010, suggesting that pollen limitation in *A. capillaris* in 2009 was probably due to pollinator scarcity rather than interspecific pollination.

The floral traits involved in the partitioning of large bees and moths in the other two species are intermediate in *A. khasiana*. Honeybees ignored *A. jasionifolia* flowers, probably because the thick and tight nectar covers prevented them from penetrating through. Bumblebees preferred *A. jasionifolia* over *A. khasiana*, probably due to the difference in nectar production between the two species. It has long been suggested that pollinator filtering can be achieved by differences in the quantity or concentration of nectar (Baker and Baker 1983). In two sympatric *Lantana* species, the less rewarding species with short floral tubes was mainly visited by short-tongued butterflies, while a more rewarding species with long floral tubes was mainly visited by long-tongued butterflies (Schemske 1976). Likewise, Gottsberger and Silberbauer-Gottsberger (2006) suggested that *Luehea grandiflora* exclusively attracted small bats and was ignored by large bats because of the small amount of nectar. This may be comparable with the partitioning of bees between *A. jasionifolia* and *A. khasiana*. Given that *A. khasiana* produced relatively little nectar and that honeybees competed for it with bumblebees, it was more rewarding for bumblebees to visit *A. jasionifolia*. Similarly, with respect to the partitioning of moth pollinators, the main nocturnal pollinators of *A. capillaris* rarely or never visited *A. khasiana*, possibly also due to less rewarding nectar and more foraging competitors on these flowers.

Spatiotemporal variation in plant–pollinator interactions

Visitor assemblages and visitation rates may exhibit substantial temporal and/or spatial variation (Herrera 1988; Schemske and Horvitz 1989; Ollerton 1996; Waser et al. 1996; Fenster and Dudash 2001; Artz et al. 2010; Reynolds et al. 2012; Zhao and Huang 2013). Pollinator categories differed between Shangri-La and Dali in *A. khasiana*, indicating that pollination in the intermediate species with less specialized flowers may be more dependent on the local ecological context. In Dali, *A. khasiana* received more visits from moths than bees. The flowers opened 3 weeks later than in Shangri-La and emitted a faint fragrance absent in the Shangri-La population, suggesting that the species may have experienced local adaptation to climate and floral visitors (Johnson 1997; Chess et al. 2008; Anderson et al. 2009; Brunet 2009; Zhao and Huang 2013).

The principal pollinators of the three *Adenophora* species did not differ across years in Shangri-La. Moreover, we observed that *A. jasionifolia* and *A. capillaris* were also visited by bumblebees and moths, respectively, as the principal pollinators at other study sites. The species composition of large bees and moths and the visitation rates varied yearly and spatially (Fig. 4), whereas a turnover of principal pollinator categories did not occur even

when the "proper" pollinators predicted by floral syndromes were scarce. Our pollination treatments excluding diurnal and/or nocturnal pollinators indicated that the roles of principal pollinators were quantitatively different among the three sympatric species (Fig. 5). Overall, our investigation of variation in flower visitor composition and pollinator effectiveness in these three sympatric species with relatively generalized flowers indicated that differences in floral traits between related species could make a major contribution to pollinator partitioning. Particularly, compared to the two other congeners, *A. khasiana* with intermediate floral traits employed two different categories of pollinators and the relative importance of diurnal and nocturnal pollinators could be shifted under different community contexts. This study highlights the importance of measuring the effectiveness of multiple pollinators to understand species coexistence in natural communities.

References

Alcantara S, Lohmann LJ (2010) Evolution of floral morphology and pollination system in Bignonieae (Bignoniaceae). Am J Bot 97:782–796

Anderson B, Alexandersson R, Johnson SD (2009) Evolution and coexistence of pollination ecotypes in an African *Gladiolus* (Iridaceae). Evolution 64:960–972

Armbruster WS (1984) The role of resin in angiosperm pollination: ecological and chemical considerations. Am J Bot 71:1149–1160

Armbruster WS (2012) Evolution and ecological implications of "specialized" pollinator rewards. In: Patiny S (ed) Evolution of plant–pollinator relationships. Cambridge University Press, Cambridge, pp 44–67

Armbruster WS, Keller S, Matsuki M, Clausen TP (1989) Pollination of *Dalechampia magnoliifolia* (Euphorbiaceae) by male euglossine bees. Am J Bot 76:1279–1285

Armbruster WS, Edwards ME, Debevec EM (1994) Floral character displacement generates assemblage structure of Western Australian trigger plants (*Stylidium*). Ecology 75:315–329

Artz DR, Villagra CA, Raguso RA (2010) Spatiotemporal variation in the reproductive ecology of two parapatric subspecies of *Oenothera cespitosa* (Onagraceae). Am J Bot 97:1498–1510

Baker HG, Baker I (1983) Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ (eds) Handbook of experimental pollination biology. Van Nostrand Reinhold, New York, pp 117–141

Botes C, Johnson SD, Cowling RM (2008) Coexistence of succulent tree aloes: partitioning of bird pollinators by floral traits and flowering. Oikos 117:875–882

Brunet J (2009) Pollinators of the Rocky Mountain columbine: temporal variation, functional groups and associations. Ann Bot 103:1567–1578

Buchmann SL (1987) The ecology of oil flowers and their bees. Annu Rev Ecol Syst 18:343–369

Chari J, Wilson P (2001) Factors limiting hybridization between *Penstemon spectabilis* and *Penstemon centranthifolius*. Can J Bot 79:1439–1448

Chess SKR, Raguso RA, LeBuhn G (2008) Geographic divergence in floral morphology and scent in *Linanthus dichotomus* (Polemoniaceae). Am J Bot 95:1652–1659

Dell'Olivo A, Hoballah ME, Gübitz T, Kuhlemeier C (2011) Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). Evolution 65:1979–1991

Erbar C, Leins P (1995) Portioned pollen release and the syndromes of secondary pollen presentation in the Campanulales-Asterales-complex. Flora 190:323–338

Fang Q, Huang S-Q (2012) Relative stability of core groups in pollination networks in a biodiversity hotspot over four years. PLoS ONE 7:e32663

Fenster CB, Dudash MR (2001) Spatiotemporal variation in the role of hummingbirds as pollinators of *Silene virginica* (Caryophyllaceae). Ecology 82:844–851

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD (2004) Pollination syndromes and floral specialization. Annu Rev Ecol Syst 35:375–403

Fulton M, Hodges SA (1999) Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. Proc R Soc Lond B 266:2247–2252

Goldblatt P, Manning JC (2006) Radiation of pollination systems in the Iridaceae of sub-Saharan Africa. Ann Bot 97:317–344

Gottsberger G, Silberbauer-Gottsberger I (2006) Life in the cerrado, a South American tropical seasonal ecosystem. Reta, Ulm

Hargreaves AL, Harder LD, Johnson SD (2010) Native pollen thieves reduce the reproductive success of a hermaphroditic plant, *Aloe maculata*. Ecology 91:1693–1703

Hentrich H, Kaiser R, Gottsberger G (2010) Floral biology and reproductive isolation by floral scent in three sympatric aroid species in French Guiana. Plant Biol 12:587–596

Herrera CM (1988) Variation in mutualisms: the spatio-temporal mosaic of a pollinator assemblage. Biol J Linn Soc 35:95–125

Herrera CM (1989) Pollinator abundance, morphology, and flower visitation rate: analysis of the "quantity" component in a plant–pollinator system. Oecologia 80:241–248

Huang SQ, Shi XQ (2013) Floral isolation in *Pedicularis*: how do congeners with shared pollinators minimize reproductive interference? New Phytol (in press)

Johnson SD (1994) Evidence for Batesian mimicry in a butterfly-pollinated orchid. Biol J Linn Soc 53:91–104

Johnson SD (1997) Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. Biol J Linn Soc 123:225–235

Johnson SD (2010) The pollination niche and its role in the diversification and maintenance of the southern African flora. Philos Trans R Soc Lond B 365:499–516

Johnson SD, Steiner KE (2000) Generalization versus specialization in plant pollination systems. Trends Ecol Evol 15:140–143

Kay KM (2006) Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. Evolution 60:538–552

Kay KM, Schemske DW (2005) Pollinator assemblages and visitation rates for 11 species of Neotropical *Costus* (Costaceae). Biotropica 35:198–207

Kessler D, Baldwin IT (2011) Back to the past for pollination biology. Curr Opin Plant Biol 14:429–434

Kingsolver JG, Daniel TL (1995) Mechanics of food handling by fluid-feeding insects. In: Chapman RF, de Boer G (eds)

Regulatory mechanisms in insect feeding. Chapman & Hall, New York, pp 32–73

Lau JA, Galloway LF (2004) Effects of low-efficiency pollinators on plant fitness and floral trait evolution in *Campanula americana* (Campanulaceae). Oecologia 141:577–583

Macior LW (1986) Floral resource sharing by bumblebees and hummingbirds in *Pedicularis* (Scrophulariaceae) pollination. Bull Torr Bot Club 113:101–109

Makholela T, Manning JC (2006) First report of moth pollination in *Struthiola ciliata* (Thymelaeaceae) in southern Africa. S Afr J Bot 72:597–603

Manning JC, Goldblatt P (2005) Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae: Crocoideae) and the development of bimodal pollination strategies. Int J Plant Sci 166:459–474

Martin NH, Willis JH (2007) Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. Evolution 61:62–82

Martins DJ, Johnson SD (2007) Hawkmoth pollination of aerangoid orchids in Kenya, with special reference to nectar sugar concentration gradients in the floral spurs. Am J Bot 94:650–659

Muchhala N (2006) The pollination biology of *Burmeistera* (Campanulaceae): specialization and syndromes. Am J Bot 93:1081–1089

Muchhala N, Potts MD (2007) Character displacement among bat-pollinated flowers of the genus *Burmeistera*: analysis of mechanism, process, and pattern. Proc R Soc Lond B 274:2731–2737

Muchhala N, Thomson JD (2012) Interspecific competition in pollination systems: costs to male fitness via pollen misplacement. Funct Ecol 26:476–482

Nilsson LA, Rabakonandrianina E, Pettersson B, Ranaivo J (1990) "Ixoroid" secondary pollen presentation and pollination by small moths in the Malagasy treelet *Ixora platythyrsa* (Rubiaceae). Plant Syst Evol 170:161–175

Olesen JM, Jordano P (2002) Geographic patterns in plant–pollinator mutualistic networks. Ecology 83:2416–2424

Ollerton J (1996) Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant–pollinator systems. J Ecol 84:767–769

Ollerton J, Killick A, Lamborn E, Watts S, Whiston M (2007) Multiple meanings and modes: on the many ways to be a generalist flower. Taxon 56:717–728

Ollerton J, Alarcón R, Waser NM, Price MV, Watts S, Cranmer L, Hingston A, Peter CI, Rotenberry J (2009) A global test of the pollination syndrome hypothesis. Ann Bot 103:1471–1480

Pauw A (2006) Floral syndromes accurately predict pollination by a specialized oil-collecting bee (*Rediviva peringueyi*, Melittidae) in a guild of South African orchids (Coryciinae). Am J Bot 93:917–926

Pérez F, Arroyo MTK, Medel R, Hershkovitz MA (2006) Ancestral reconstruction of flower morphology and pollination systems in *Schizanthus* (Solanaceae). Am J Bot 93:1029–1038

Ramsey J, Bradshaw HD, Schemske DW (2003) Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520–1534

Reynolds RJ, Westbrook MJ, Rohde AS, Cridland JM, Fenster CB, Dudash MR (2009) Pollinator specialization and pollination syndromes of three related North American *Silene*. Ecology 90:2077–2087

Reynolds RJ, Kula AAR, Fenster CB, Dudash MR (2012) Variable nursery pollinator importance and its effect on plant reproductive success. Oecologia 168:439–448

Roquet C, Sáez L, Aldasoro JJ, Susanna A, Alarcón ML, Garcia-Jacas N (2008) Natural delineation, molecular phylogeny and floral evolution in *Campanula*. Syst Bot 33:203–217

Sakamoto RL, Ito M, Kawakubo N (2012) Contribution of pollinators to seed production as revealed by differential pollinator

exclusion in *Clerodendrum trichotomum* (Lamiaceae). PLoS ONE 7:e33803

Schemske DW (1976) Pollinator specificity in *Lantana camara* and *L. trifolia* (Verbenaceae). Biotropica 176:260–264

Schemske DW, Horvitz CC (1989) Temporal variation in selection on a floral character. Evolution 43:461–465

Schiestl FP, Schlüter PM (2009) Floral isolation, specialized pollination, and pollinator behavior in orchids. Annu Rev Entomol 54:425–446

Schiestl FP, Ayasse M, Paulus HF, Löfstedt C, Hansson BS, Ibarra F, Francke W (1999) Orchid pollination by sexual swindle. Nature 399:421–422

Stone GN, Willmer P, Rowe JA (1998) Partitioning of pollinators during flowering in an African *Acacia* community. Ecology 79:2808–2827

Thomson JD, Wilson P (2008) Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. Int J Plant Sci 169:23–38

Thomson JD, Wilson P, Valenzuela M, Malzone M (2000) Pollen presentation and pollination syndromes, with special reference to *Penstemon*. Plant Spec Biol 15:11–29

van der Niet TS, Johnson SD (2012) Phylogenetic evidence for pollinator-driven diversification of angiosperms. Trends Ecol Evol 27:353–361

Waelti MO, Muhlemann JK, Widmer A, Schiestl FP (2008) Floral odour and reproductive isolation in two species of *Silene*. J Evol Biol 21:111–121

Waser NM, Chittka L, Price MV, Williams NM, Ollerton J (1996) Generalization in pollination systems, and why it matters. Ecology 77:1043–1060

Wilson P, Castellanos MC, Wolfe AD, Thomson JD (2006) Shifts between bee and bird pollination in *Penstemon*. In: Waser NM, Ollerton J (eds) Plant–pollinator interactions: from specialization to generalization. University of Chicago Press, Chicago, pp 47–68

Wolfe LM, Sowell DR (2006) Do pollination syndromes partition the pollinator community? A test using four sympatric morning glory species. Int J Plant Sci 167:1169–1175

Zhao Z-G, Huang S-Q (2013) Differentiation of floral traits associated with pollinator preference in a generalist-pollinated herb, *Trollius ranunculoides* (Ranunculaceae). Int J Plant Sci 174:637–646