Balsaminaceae in Southeast Asia: systematics, evolution, and pollination biology

Ruchisansakun, S.

Citation

Ruchisansakun, S. (2018, September 19). *Balsaminaceae in Southeast Asia: systematics, evolution, and pollination biology*. Retrieved from https://hdl.handle.net/1887/65602

Author: Ruchisansakun, S.
Title: Balsaminaceae in Southeast Asia: systematics, evolution, and pollination biology
Issue Date: 2018-09-19

CHAPTER 6

Floral specialization for different pollinators and divergent use of the same pollinator among co-occurring *Impatiens* species (Balsaminaceae) from Southeast Asia

Saroj Ruchisansakun[1, 2], Pornpimon Tangtorwongsakul[3], Ruth J. Cozien[1, 4], Erik F. Smets[1, 2, 5], Timotheüs van der Niet[1, 2, 4]

[1] Naturalis Biodiversity Center, PO Box 9517, 2300 RA Leiden, the Netherlands

[2] Leiden University, PO Box 9517, 2300 RA Leiden, the Netherlands

[3] King Mongkut's University of Technology Thonburi, 83 Moo 8, Takham, Bangkhuntien, 10150, Bangkok, Thailand

[4] School of Life Sciences, University of KwaZulu-Natal, P. Bag X01, 3209 Scottsville, South Africa

[5] Section Ecology, Evolution and Biodiversity Conservation, KU Leuven, BE-3001 Leuven, Belgium

Published in: Botanical Journal of the Linnean Society, 181 (2016): 651–666.

6.1 ABSTRACT

Floral variation among closely related species is thought to often reflect differences in pollination systems. Flowers of the large genus *Impatiens* are characterized by extensive variation in colour, shape and size and in anther and stigma positioning, but studies of their pollination ecology are scarce and most lack a comparative context. Consequently, the function of floral diversity in *Impatiens* remains enigmatic. This study documents floral variation and pollination of seven co-occurring *Impatiens* spp. in the Southeast Asian diversity hotspot. To assess whether floral trait variation reflects specialization for different pollination systems, we tested whether species depend on pollinators for reproduction, identified animals that visit flowers, determined whether these visitors play a role in pollination and quantified and compared key floral traits, including floral dimensions and nectar characteristics. Experimental exclusion of insects decreased fruit and seed set significantly for all species except *I. muscicola*, which also received almost no visits from animals. Most species received visits from several animals, including bees, birds, butterflies and hawkmoths, only a subset of which were effective pollinators. *Impatiens psittacina*, *I. kerriae*, *I. racemosa* and *I. daraneenae* were pollinated by bees, primarily Bombus haemorrhoidalis. *Impatiens chiangdaoensis* and *I. santisukii* had bimodal pollination systems which combined bee and lepidopteran pollination. Floral traits differed significantly among species with different pollination systems. Autogamous flowers were small and spurless, and did not produce nectar; bee-pollinated flowers had short spurs and large floral chambers with a wide entrance; and bimodally bee- and lepidopteran-pollinated species had long spurs and a small floral chamber with a narrow entrance. Nectar-producing species with different pollination systems did not differ in nectar volume and sugar concentration. Despite the high frequency of bee pollination in co-occurring species, individuals with a morphology suggestive of hybrid origin were rare. Variation in floral architecture, including various forms of corolla asymmetry, facilitates distinct, species-specific pollen-placement on visiting bees. Our results show that floral morphological diversity among *Impatiens* spp. is associated with both differences in functional pollinator groups and divergent use of the same pollinator. Nonhomologous mechanisms of floral asymmetry are consistent with repeated independent evolution, suggesting that competitive interactions among species with the same pollination system have been an important driver of floral variation among *Impatiens* spp.

6.2 INTRODUCTION

Diversity in traits among angiosperm flowers has frequently been explained in the context of plant–pollinator interactions (Darwin, 1862). In particular, repeated broad-scale similarities in floral traits among distantly related plant species have been interpreted as evidence for widespread specialization for different functional pollinator groups (Vogel, 1954; Faegri & van der Pijl, 1979; Fenster *et al.*, 2004). However, evidence from studies that have quantified floral visitation by animals has suggested that plant species thought to be specialized for a particular functional pollinator group are often visited by a suite of animals representing multiple functional groups (Waser *et al.*, 1996) or by representatives from a different functional pollinator group than implied by floral traits (e.g. Fishbein & Venable, 1996; Mayfield, Waser & Price, 2001). Furthermore, evidence has accumulated that floral traits also perform functions that are unrelated to pollination, such as mediating interactions between plants and herbivores or the abiotic environment (e.g. Strauss & Whittall, 2006; Wang *et al.*, 2013; De Jager & Ellis, 2014). Hence, confirmation of the link between floral diversity and specialization for different functional pollinator groups requires a combination of analyses of floral traits and detailed observations to distinguish visitors and pollinators.

Associations between floral diversity and specialized pollination may differ geographically (Johnson & Steiner, 2003; Pauw & Stanway, 2015). For example, in temperate floras, environmental perturbations during glacial cycles might have favoured generalized plant–pollinator interactions, whereas in tropical regions, relatively stable climates might allow for persistence of highly specialized biotic interactions (e.g. Schemske *et al.*, 2009; Dalsgaard *et al.*, 2011; but see Schleuning *et al.*, 2012). Furthermore, some researchers have predicted that competitive biotic interactions play an important role in determining high levels of ecological specialization in biodiverse tropical regions at lower latitudes (e.g. Pianka, 1966), although absence of latitudinal trends (Ollerton & Cranmer, 2002) or opposite trends (Schleuning *et al.*, 2012) have also been reported. More research is required to assess whether specialized pollination systems, associated with floral diversity, are common in tropical ecosystems (Ollerton, 2012). The majority of studies addressing the potential link between floral diversity and pollination ecology, however, focus on temperate regions (e.g. Grant & Grant, 1965; Goldblatt & Manning, 2006; Johnson *et al.*, 2011; Valente *et al.*, 2012; Armbruster, Shi & Huang, 2014; Van der Niet *et al.*, 2014) or are mostly limited to the tropics in the

New World and Madagascar (e.g. Armbruster & Herzig, 1984; Freitas *et al*., 2006; Smith *et. al*., 2008; Tripp & Manos, 2008).

Impatiens L. (Balsaminaceae) consists of >1000 species distributed mainly in tropical regions of the Old World (Grey-Wilson, 1980; Yuan *et al*., 2004; Janssens *et al*., 2009; Yu *et al*., 2015). The typically unscented flowers vary extensively in shape, colour, presence and length of the spur and nectar properties (Vogel, 1954; Grey-Wilson, 1980; Kato *et al*., 1991; Janecek, Bartos & Njabo, 2015). Variation in these traits has been shown to be important for mediating specialized interactions with different functional pollinator groups in other plant groups (e.g. Baker & Baker, 1983; Whittall & Hodges, 2007; Van der Niet *et al*., 2014). A relationship between floral morphology of (predominantly African) *Impatiens* spp. and their pollinator type has been suggested by Vogel (1954) and Grey-Wilson (1980). Although terminology differed, both emphasized variation in sepal, petal and spur morphology and flower colour as being important for identification of distinct flower types pollinated by bees, moths, butterflies and birds, respectively. Grey-Wilson (1980) suggested that ‘flat’ pale to deep pink flowers with shallow lower sepals, a long spur and a narrow entrance are probably pollinated by butterflies; yellow, white or pale pink funnel-shaped flowers, with deep and wide lower sepals, hood-like dorsal petals (i.e. a wide entrance), and short spurs are probably bee-pollinated; red or orange flowers with a broad entrance would be bird-pollinated; and white funnel-shaped flowers with a long spur are probably moth-pollinated. However, few studies have collected the empirical data required to verify these predictions. Fragmentary evidence suggests that African *Impatiens* spp. with red flowers and trumpet-shaped lower sepals are specialized for sunbird pollination (Bartos & Janecek, 2014; Janecek *et al*., 2015), whereas most studies performed outside Africa suggest pollination by bees (Rust, 1977; Schemske, 1978; Kato, 1988; Kato *et al*., 1991; Wilson, 1995; Tian, Liu & Hu, 2004; Sreekala *et al*., 2008, 2011; Ramasubbu *et al*., 2011; Mohandass, 2013). Some Asian species, such as *I. reptans* Hook.f. and *I. cuspidata* Wight & Arn., are characterized by relatively generalized pollination systems, including pollination by hawkmoths, bees and butterflies (Tian *et al*., 2004; Sreekala *et al*., 2011). Thus, some evidence from observations to date contradicts the hypothesis that variation among *Impatiens* flowers reflects different, specialized pollination systems. Progress in resolving whether floral variation indeed reflects differences in pollination would be aided by studying *Impatiens* spp. in a comparative context, similar to previous studies, for instance, in Iridaceae (Goldblatt & Manning, 2006) and *Aquilegia* L. (Whittall & Hodges, 2007). In particular, comparative studies that are performed in a restricted area are useful, because these can distinguish specialization due to partitioning of a local pollinator assemblage from apparent specialization due to geographical turnover in pollinator assemblages. The aim of this study was therefore to assess whether floral diversity of seven *Impatiens* spp. from Southeast Asia, which occur in close proximity, is associated with different, specialized pollination systems. We first determined whether plants require animals for pollination. We then observed visitors to identify whether species are specialized for different functional pollinator groups. Finally, we assessed whether similarity in floral characters is associated with similarity in pollination systems.

6.3. MATERIAL AND METHODS

6.3.1 Study area and study species

This study was performed on Doi Chiang Dao Mountain in the Chiang Dao Wildlife Sanctuary (c. 521 km2) in the Chiang Mai province of Thailand during November and December 2014. Doi Chiang Dao is the highest limestone mountain in Thailand (elevation 2275 m). Eight *Impatiens* spp. are known from this area; *I. chiangdaoensis* T.Shimizu, *I. daraneenae* Suksathan&Triboun and *I. muscicola* Craib are endemic to the reserve, *I. santisukii* T.Shimizu is endemic to Thailand and *I. discolor* DC., *I. kerriae* Craib, *I. psittacina* Hook.f. and *I. racemosa* DC. have a broader range in Southeast Asia. All species known from the reserve, apart from *I. discolor*, which did not flower due to frost damage, were studied. Six studied species are distributed in lower montane mixed deciduous forest to upper montane scrub, between latitudes 19°22036.47″ N and 19°23055.77″ N, longitudes 98°5008.78″ E and 98°53030.78″ E and 1350–2225 m (Fig. 6.1), whereas *I. daraneenae* occurs at lower elevations in mixed deciduous forests, around 19°23044.73″ N and 98°46028.70″ E at 768 m, c. 6.7 km from the nearest population of the other species (Fig. 6.1). All species are annual herbs, apart from *I. kerriae*, which is a perennial shrub. Flower longevity is 3 to 4 days for *I. daraneenae*, *I. kerriae*, and *I. psittacina* and 5 to 6 days for *I. chiangdaoensis*, *I. muscicola*, and *I. santisukii* (flower longevity of *I. racemosa* is unknown).

To document whether and which species co-flower, and hence are exposed to the same pollinator assemblage, we recorded the occurrence of flowering populations of all species along a walking trail that includes a cross-section of the habitat types and elevation zones in the reserve. GPS coordinates were recorded for each patch of flowering *Impatiens* spp. throughout the study period from the end of the rainy season to the start of the winter season, during November and December

2014. Whether co-flowering species are exposed to the same pollinator species depends on the maximum flight distances of pollinators. For species included in this study, such data are unavailable. However, given that the most common pollinator in our study was an Asian bumble bee species (recorded for five out of seven species), we used the maximum foraging distance of European bumble bees as a proxy (Wolf & Moritz, 2008). Species were therefore recorded as co-occurring if the distance between patches was < 800 metres.

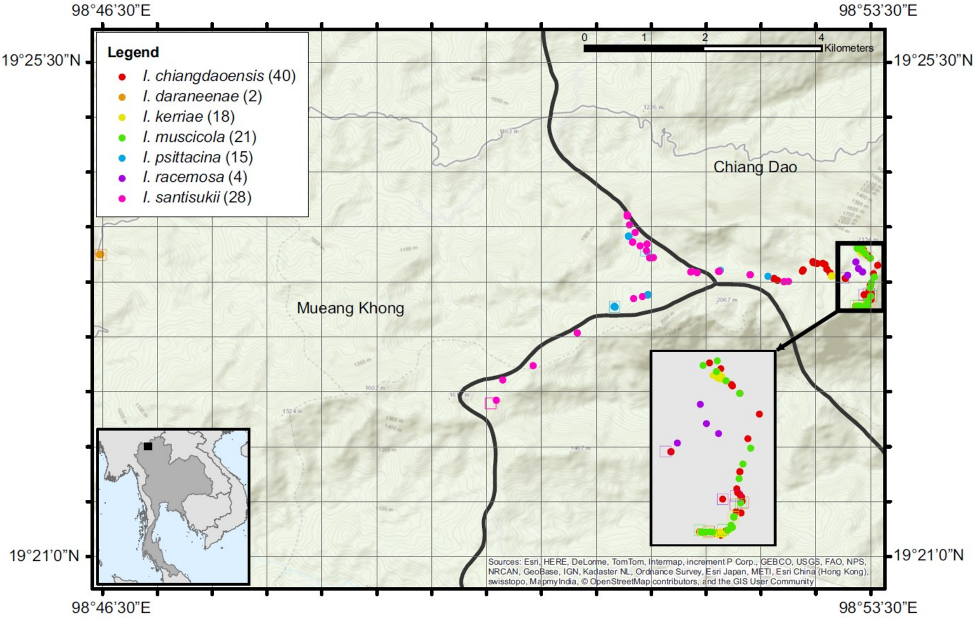

Fig. 6.1. The distribution of studied *Impatiens* spp. in the Chiang Dao Wildlife Sanctuary along a walking trail. Numbers between brackets indicate the number of patches found. Study sites for each species are indicated with squares.

6.3.2. Visitor exclusion experiment

To test whether plants rely on pollinators for reproduction we performed a visitor exclusion experiment. In one population per species, 7 to 12 (median = 11) plants of each species were selected randomly. On each plant, two buds of similar age, just prior to anthesis, were identified and assigned randomly to an exclusion treatment or an unmanipulated control. The treatment bud was covered with a mesh bag (mesh diameter < 1 mm). After all floral parts apart from the gynoecium were shed, (c. 3–4 days) both flowers were bagged to prevent loss of seeds through explosive dispersal. Once flowers had wilted after c. 4 weeks, the presence or absence of a fruit capsule was recorded and the number of seeds was counted for all developed fruits. To establish the effect of visitor exclusion on fecundity, fruit and seed set were analysed using generalized linear models with a binomial distribution and logit link function and a negative binomial error structures and log link function, respectively, implemented in SPSS 22 (IBM Corp., Armonk, NY, USA). Means and standard errors were back-transformed from the logit and log scale, respectively, for graphical presentation.

6.3.3. Floral visitor observations

To identify floral visitors, quantify visitation rates and assess the potential role of visitors as pollinators, floral visitors and their behaviour were studied by direct observations. Observations were conducted from half an hour before sunrise until half an hour after sunset, for 2 to 3 days per species in two to four populations in the study area (20–44 h observation for each species, median 37.5 h) (Fig. 6.1). Flowers of *I. racemosa* in the Chiang Dao Wildlife Sanctuary were already wilting during the study period. Additional visitor observations for this species were therefore performed on 23 November 2014 between 12.00 and 15.00 in a similar habitat in the Doi Inthanon National Park, 18°33009.3″ N 98°28047.2″ E, 2142 m elevation, 120 km from the Chiang Dao Wildlife Sanctuary. All visits to flowers were noted and visitor species were identified to the lowest taxonomic level possible. To determine whether visitors contact plant reproductive parts, visits were photographed and video-recorded using a Canon 60D camera (Tokyo, Japan) and representative visitors were captured with a hand-held insect net, immobilized by anaesthetic ether and checked for the presence of pollen. To summarize visitation rates, visitor species were categorized according to functional groups (cf. Fenster *et al*., 2004). Animal species representing the same order are expected to functionally resemble each other in perceptional bias and morphology. Potential pollinator specialization is therefore expected to mostly occur at the level of functional groups, rather than at the level of individual animal species (Johnson & Steiner, 2003; Fenster *et al*., 2004). Following Vogel (1954) and Grey-Wilson (1980), five functional groups were used: bee; bird; butterfly; day-flying hawkmoth; and other (including infrequent visitors of diverse taxonomic origin). Visitation rates for each functional group were calculated by dividing the total number of visits by the total number of hours that a particular *Impatiens* spp. had been observed. To determine the functional pollinator group(s) of each *Impatiens* spp., we excluded visitors that did not contact the anther and stigma. As we could not establish whether day-flying hawkmoths contacted the anther and stigma during visits, we established the broadly defined functional group Lepidoptera for this purpose, to accommodate the possibility that day-flying hawkmoths are effective pollinators besides butterflies.

6.3.4. Floral trait variation

To establish whether floral trait variation is associated with different pollination systems, five key floral traits which are considered to be important for pollination were quantified. For one flower sampled randomly from seven to ten (mean = 7.8) individuals of each species, floral entrance width (gap between lateral united petals), spur length, lower sepal length, lower sepal width and lower sepal height (see Fig. 6.S1 for diagrammatic representation of each character) were measured to the nearest 0.1 mm using Vernier calipers. *Impatiens racemosa* was not measured due to wilting in the focal population. Flower colour was scored on the basis of visual inspection of flowering plants. Mechanisms of floral asymmetry were described from dissected flowers.

Variation in all measured quantitative characters was visualized using dimension reduction based on principal component analysis (PCA) in the program PAST version 3.02a (Hammer, Harper & Ryan, 2001). We first calculated a variance–covariance matrix based on the quantitative characters and plotted the first two principal components. To test for an association between floral traits and pollination systems, we performed a nested two-way ANOSIM (Clarke, 1993) with species nested in pollination system, defined based on the functional pollinator group(s) of each species (see Results section). ANOSIM is a non-parametric test that evaluates whether dissimilarity in traits within groups is smaller than between two or more groups, based on a distance matrix (Clarke, 1993). Groups were compared based on the Euclidean distance of morphological measurements of species. To calculate significance, samples were randomly assigned to groups 9999 times and the observed dissimilarity was contrasted against the generated distribution of dissimilarity values in Primer Version 6.1.6 (Clarke & Gorley, 2006). To identify which characters contribute most to dissimilarity within and among predefined groups based on pollination systems (see results), we calculated the similarity percentage based on Euclidean distances of morphological characters in a SIMPER analysis (Clarke, 1993) in Primer Version 6.1.6 (Clarke & Gorley, 2006).

Nectar properties were measured from one flower of five to ten individuals (median = 6.5) of each species. To measure the total volume of nectar available for pollinators at anthesis, flowers were bagged before anthesis and nectar volume was measured 1 day after anthesis at 16:00 h. Nectar volume was measured by cutting the tip of the spur and gently squeezing nectar into glass microcapillary tubes [volume 1–5 lL, appropriate for all studied species (Drummond Scientific Company, Broomall, PA, USA)]. Nectar sugar concentration (% w/w) was measured with a handheld refractometer (MASTER-53a Atago, Japan). To assess whether differences in pollination system are associated with differences in nectar properties among the studied *Impatiens* spp., the effect of the predefined pollination systems (fixed factor, see Results) on nectar volume and concentration, respectively, was modelled using REML in the MIXED procedure in SPSS 22 (IBM Inc.). Species nested within pollination system was included as a random factor to account for correlation among measurements of multiple flowers of each species. *Impatiens muscicola*, which does not produce any nectar, was excluded from these analyses.

6.4. RESULTS

6.4.1. Visitor exclusion experiment

With the exception of *I. muscicola*, fruit and seed sets were significantly lower for flowers from which animals were excluded compared with unmanipulated control flowers for all species (Fig. 6.2). In five species, no fruits were developed in any of the flowers from which visiting animals were excluded (Fig.6. 2).

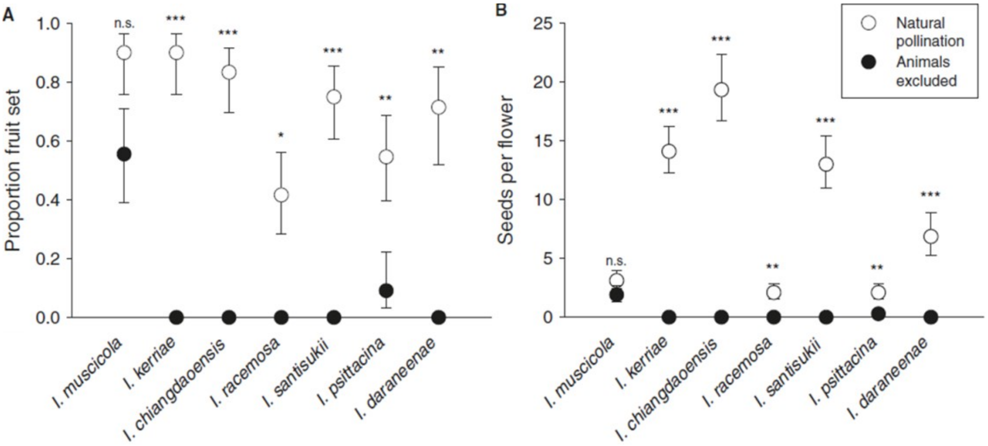

Fig. 6.2. Comparison of proportion fruit set (A) and the number of seeds per flower (B) between open-pollinated flowers and flowers of Impatiens spp. from which animal visitors were excluded. Stars indicate the level of significance for intraspecific comparisons ***P < 0.001, **P < 0.01, *P < 0.05, n.s., not significant.

6.4.2. Floral visitor observations

During 251.5 h of observations, 461 visits to *Impatiens* flowers by 14 different animal species were observed (Table 6.1). Visitor species included four bee species, one bird species, four butterfly species, one day-flying hawk moth species and four species grouped as 'other': one ant species; one cockroach species; one beetle species; and one fly species (Fig. 6.3; Table 6.1). Most *Impatiens* spp. were visited by more than one animal species (Figs 6.3, 6.4; Table 6.1).

Table 6.1. Floral visitors, behaviour, pollen placement and visitation rates to seven *Impatiens* spp.

***Impatiens* species**	**Visitor species**	**Behavior**	**Pollen placement site**	**Visits/hour (number of hours observed)**	**Functional Pollinator group**
I. muscicola	Hymenoptera: Formicidae: *Tetraponra nigra*	Non-pollinating visitor	Unspecific	0.05 (44)	N/A
	Dictyoptera: Blattodea: unknown sp.	Non-pollinating visitor	Unspecific	0.05 (44)	
I. kerriae	Hymenoptera: Apidae: *Bombus haemorrhoidalis*	Pollinator	Left side of body	2.10 (39)	Bees
	Aves: Nectariniidae: *Aethopyga gouldiae*	Nectar robber	N/A	0.59 (39)	
	Hymenoptera: Apidae : *Apis cerana*	Nectar robber	N/A	0.15 (39)	
	Lepidoptera: Sphingidae: *Macroglossum belis* L.	Unknown	Unknown	0.05 (39)	
I. racemosa	Hymenoptera: Apidae : *Bombus haemorrhoidalis*	Pollinator	Head	0.05 (39)	Bees
I. chiangdaoensis	Lepidoptera: Sphingidae: *Macroglossum belis*	Unknown	Unknown	0.40 (37.5)	Bimodal: Bees and Lepidoptera
	Hymenoptera: Apidae : *Bombus haemorrhoidalis*	Pollinator Nectar robber	Head N/A	0.37 (37.5) 0.16 (37.5)	
	Coleoptera: unknown sp.	Non-pollinating visitor	Unspecific	0.21 (37.5)	
	Lepidoptera: Hesperiidae: *Notocrypta curvifascia*	Pollinator	Proboscis	0.19 (37.5)	
	Diptera: Drosophilidae: unknown sp.	Non-pollinating visitor	Unspecific	0.08 (37.5)	
	Hymenoptera: Apidae: *Apis cerana*	Pollinator	Head	0.03 (37.5)	
I. psittacina	Hymenoptera: Apidae: *Bombus haemorrhoidalis*	Pollinator Nectar robber	Dorsal thorax	3.55 (20) 2.45 (20)	Bees
	Lepidoptera: Hesperiidae: unknown sp.	Unknown	Unknown	0.05 (20)	
	Lepidoptera: Sphingidae: *Macroglossum belis*	Unknown	Unknown	0.05 (20)	

***Impatiens* species**	**Visitor species**	**Behavior**	**Pollen placement site**	**Visits/hour (number of hours observed)**	**Functional Pollinator group**
I. santisukii	Lepidoptera: Hesperiidae : *Polytremis discreta discreta*	Pollinator	Proboscis	0.94 (36)	Bimodal: Bees and Lepidoptera
	Lepidoptera: Hesperiidae : *Polytremis lubricans lubricans* Herrich-Schäffer	Pollinator	Proboscis	0.31 (36)	
	Lepidoptera: Sphingidae: *Macroglossum belis*	Unknown	Unknown	0.28 (36)	
	Hymenoptera: Apidae: *Bombus haemorrhoidalis*	Pollinator	Head	0.28 (36)	
I. daraneenae	Hymenoptera: Apidae: unknown sp.	Pollinator	Right legs	1.42 (36)	Bees
	Lepidoptera: Hesperiidae: *Notocrypta curvifascia*	Nectar robber	N/A	0.61 (36)	
	Hymenoptera: Apidae: *Tetragonula* sp.	Pollen robber	N/A	0.55 (36)	
	Hymenoptera: Halictidae or Megachilidae: unknown sp.	Pollen robber	N/A	0.22 (36)	

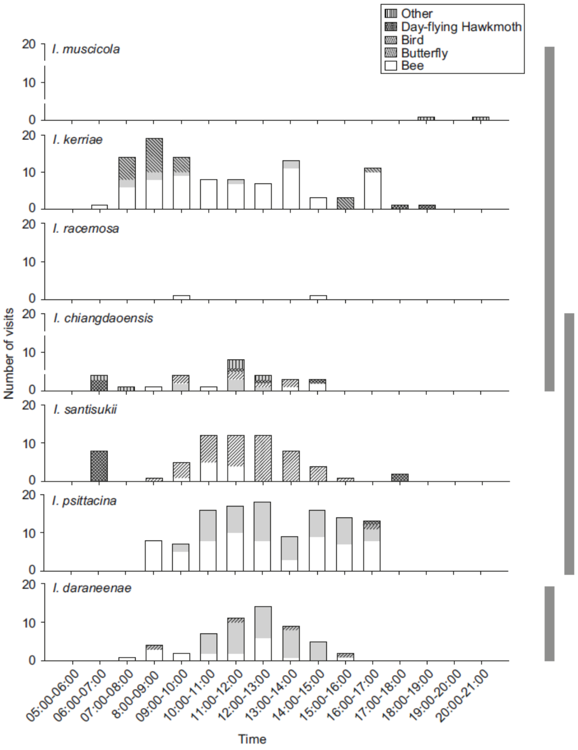

Fig. 6.4. Visitation rates per hour by five functional pollinator groups to studied *Impatiens* spp. White shading indicates pollinating visits, grey shading indicates robbing visits or day-flying hawkmoth visits for which resulting pollination could not be confirmed. Perpendicular bars on the right-hand side indicate co-occurrence of species within 800 m in the Chiang Dao Wildlife Sanctuary.

Fig. 6.3. *Impatiens* flowers, showing variation in colour and shape and floral visitors: *I. muscicola* (A); *I. santisukii* pollinated by *P. lubricans lubricans* (B) and *B. haemorrhoidalis* (C); *I. racemosa* pollinated by *B. haemorrhoidalis* (D); *I. chiangdaoensis* pollinated by *N. curvifascia* (E) and visited by a nectar-robbing *B. haemorrhoidalis* (F); *I. psittacina*

During 44 h of observation on *I. muscicola*, only two visits by ants (*Tetraponera nigra*) and two by cockroaches, all after sunset, were recorded (Fig. 6.4; Table 6.1). Although pollen was present on the body of the cockroach (Video S1), it did not appear to visit flowers for foraging and seldom moved between flowers. No visitors were, therefore, considered effective pollinators of *I. muscicola* (Table 6.1).

In the same area as *I. muscicola*, *I. kerriae* was most frequently visited by bumblebees (*Bombus haemorrhoidalis*) throughout the day (mean visitation rate: 2.10 visits/h) (Figs 6.3, 6.4; Table 6.1). Bumblebees moved among flowers during foraging bouts and consistently contacted the anthers or stigmas and were therefore considered to be effective pollinators. The flower of *I. kerriae* is asymmetrical; the left-hand side of the lower lateral petal is slightly uplifted relative to the right-hand side, resulting in a wider entrance to the flower on the right-hand side. Bumblebees always entered flowers on the right-hand side and carried pollen consistently on the left-hand side of their upper thorax and wing (Figs 6.3, 6.5; Video S2). *Impatiens kerriae* also received visits from Mrs Gould's sunbird, *Aethopyga gouldiae*, which robbed nectar by piercing a hole in the outside of the spur (mean visitation rate: 0.59 visits/h) (Figs 6.3, 6.4; Video S1). Asian honey bees, *Apis cerana*, foraged for nectar (mean visitation rate: 0.15 visits/h) in the same way as bumblebees, but did not touch the anther due to their small size relative to the flowers (Figs 6.3, 6.4; Video S1). Day-flying hawkmoths, *Macroglossum belis*, visited flowers infrequently (mean visitation rate: 0.05 visits/h) during a short period before sunset (Figs 6.3, 6.4). Hawkmoths hovered in front of flowers while inserting the proboscis into the spur. Due to the rapid movement of *M. belis* it could not be confirmed whether it is an effective pollinator. Due to the low visitation rates of day-flying hawkmoths (1.7% of all visits), we considered only bees to be effective pollinators of *I. kerriae* (Table 6.1).

On *I. racemosa*, only two visits by bumblebees (*B. haemorrhoidalis*) were observed in the Chiang Dao Wildlife Sanctuary (mean visitation rate: 0.05 visits/h) (Fig. 6.4; Table 6.1). However, frequent visits by bumblebees (*B. haemorrhoidalis*) to *I. racemosa* were observed in Doi Inthanon (mean visitation rate: 6.00 visits/h) (Fig. 6.3). Bumblebees always landed on the lower lateral petals and probed the spur for nectar by inserting the proboscis through the entrance gap between the lateral united petals. Pollen was attached on the bumblebee head (Figs 6.3, 6.5; Video S2). Thus, only bees were considered as effective pollinators of *I. racemosa* (Table 6.1).

Impatiens chiangdaoensis was visited by six animal species (Fig. 6.4; Table 6.1). Day-flying hawkmoths, *M. belis*, frequently visited half an hour after sunrise and occasionally during the day in cloudy conditions (mean visitation rate: 0.40 visits/h) (Fig. 6.4). Hawkmoths probed flowers by inserting their long proboscides into the long floral spur to consume nectar and moved quickly among flowers. The rapid movement of hawkmoths prevented confirmation of their effectiveness as pollinators. Bumblebees (*B. haemorrhoidalis*) were the next most frequent day-time visitors (mean visitation rate: 0.37 visits/h) (Fig. 6.4). Pollen was deposited on the head when bumblebees entered flowers (Fig. 6.5). In approximately one-third of visits, bumblebees robbed nectar by biting a hole in the spur (Fig. 6.3; Table 6.1). Bristle beetles (order Coleoptera) were also often observed as floral visitors; these carried pollen on their wings, but seldom moved between flowers (mean visitation rate: 0.21 visits/h) (Fig. 6.4). Restricted demon butterflies, *Notocrypta curvifascia*, visited flowers during day time (mean visitation rate: 0.19 visits/h) (Figs 6.3, 6.4), landing on the lateral united petals and probing the spur with their long proboscides. Pollen was deposited on the proboscis (Fig. 6.3; Video S2). Fruit flies (Drosophilidae) were observed visiting, but seldom moved between the flowers (mean visitation rate: 0.08 visits/hour) or carried pollen (Fig. 6.4). Finally, Asian honey bees (*A. cerana*) visited flowers at a low frequency (mean visitation rate: 0.03 visits/h) (Table 6.1); pollen was attached on the head. *Impatiens chiangdaoensis* is characterized by a bimodal pollination system, including pollination by bees and Lepidoptera (Table 6.1).

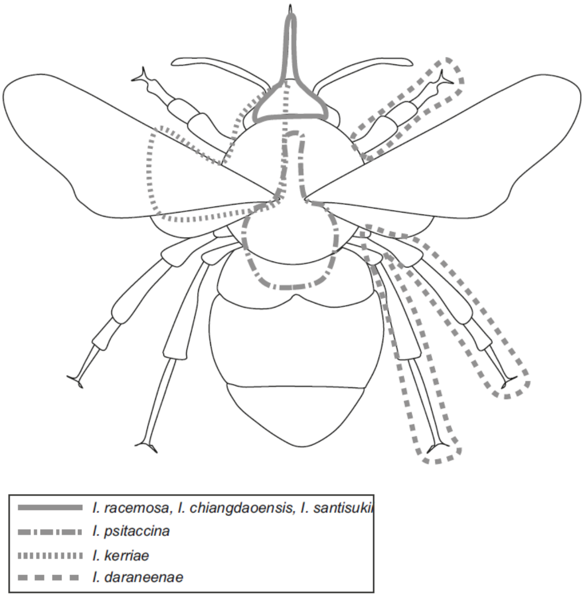

Fig. 6.5. Diagram of a bee body showing pollen placement for six *Impatiens* spp. that are (partly) bee pollinated. The most common bee pollinator is *B. haemorrhoidalis* and *I. dararneenae* is predominantly pollinated by an unknown bee (Apidae).

Impatiens psittacina was visited by bumblebees (*B. haemorrhoidalis*) during daylight hours (mean visitation rate: 6.00 visits/h) (Figs 6.3, 6.4). Bumblebees landed on the lower lobe of the lateral united petals and crawled into the flowers to forage nectar from the spur. When departing a flower, bumblebees faced the direction of the spur, so that pollen was placed on the dorsal thorax under the wings, which unfolded as the bee moved backwards (Fig. 6.5; Video S2). In 41% of visits, bumblebees landed close to the spur on the outside of the flower and pierced through the apex of the spur to drink nectar without pollinating flowers (Video S1). During 20 h of observation, one visit by an unknown butterfly species and one by a day-flying hawkmoth were also recorded. Contact between these insects and plant reproductive parts could not be assessed. Due to the low relative visitation rate of Lepidoptera (1.6%), only bees were considered pollinators of *I. psittacina* (Table 6.1).

Impatiens santisukii was most frequently visited by two butterfly species; the Himalayan swift butterfly, *Polytremis discreta discreta*, and the contiguous swift butterfly, *Polytremis lubricans lubricans*, which visited flowers at a mean rate of 0.94 visits/h and 0.31 visits/h, respectively, during daylight hours (Fig. 6.4; Table 6.1). Butterflies always landed on the lateral united petals and used their extended proboscides to consume nectar from the long spur (Fig. 6.3; Video S2). Pollen was placed on the proboscis. The day-flying hawkmoth, *M. belis*, also visited flowers immediately after sunrise and immediately before sunset (mean visitation rate: of 0.28 visits/h) (Fig. 6.4), but it could not be determined whether the hawkmoth carried pollen. Bumblebees (*B. haemorrhoidalis*) visited flowers at a mean visitation rate of 0.28 visits/h (Fig. 6.4). They landed on the lateral united petals and probed the spur to consume nectar by inserting the proboscis through the floral entrance (a small gap between the lateral united petals). Pollen was attached on the head of bumblebees (Figs 6.3, 6.5; Video S2). *Impatiens santisukii* is characterized by a bimodal pollination system, including pollination by bees and Lepidoptera (Table 6.1).

Impatiens daraneenae was visited by four insect species (Table 6.1). An unknown bee species (Apidae) was the most frequent visitor (mean visitation rate: 1.42 visits/h) (Fig. 6.4). The flower of *I. daraneenae* is asymmetrical (Fig. 6.3): the lateral united petals are distorted counter-clockwise, such that pollen is placed on the right legs of a bee as it enters a flower (Figs 6.3, 6.5; Video S2). The restricted demon butterfly, *N. curvifascia*, also visited flowers (mean visitation rate: 0.61 visits/h) (Fig. 6.4). These butterflies landed on the lateral united petals and they probed the spur for nectar by inserting their long proboscides through the wide floral entrance, but did not contact anther or stigma. Other bee species, *Tetragonula* sp. and an unknown bee species (Megachilidae), visited only male-stage flowers to collect pollen; these were therefore not considered pollinators (Table 6.1, Video S1). Bees were considered the only pollinator of *I. daraneenae* (Table 6.1).

6.4.3. Floral trait variation

The flowers of each studied *Impatiens* spp. differ in shape and colour (Fig. 6.3). Species cluster by predefined pollination categories based on functional pollinator group(s) (Table 6.1; Autonomous selfing, Bee pollination, Bee + Lepidoptera pollination) in multivariate space of floral trait variation (Fig. 6.6). The first two PC axes explained 89.9% of trait variance. PC 1 explained 71.7% of variation and correlated positively with all traits except spur length. PC 2 explained 18.2% of variation and correlated positively with spur length (Fig. 6.6). Floral traits were significantly more similar within than between pollination systems (Fig. 6.6).

Bee-pollinated species differed primarily in sepal dimensions (see Table 6.S1. for all SIMPER results; 85.7% of variation; height 42%, width 24.2%, length 19.5%), whereas flower entrance size (9.6%) and spur length (4.7%) were more consistent. In contrast, differences in spur length characterized the most (87.6%) variation between the two bimodally (Bee and Lepidoptera) pollinated species, relative to more consistent sepal morphology (11.3%) and flower entrance dimensions (1.1%). Among species with different pollination systems, autogamous species differed from bee-pollinated species primarily in sepal dimensions (87.9%, of which length 39.2%, height 30.1%, width 18.6%), and from bimodally (bee and Lepidoptera) pollinated species primarily in spur length (87.0%). Flower entrance (14.2%), sepal dimensions (height 25.1%, length 27.5%, width 13.6%) and spur length (19.6%) varied to a comparable extent between bee-pollinated and bimodally (bee and Lepidoptera) pollinated species.

With the exception of *I. muscicola*, in which no nectar was found, mean nectar volume ranged between 1.89 lL (*I. daraneenae*) to 14.45 lL (*I. santisukii*) and sugar concentration between 32.7 and 39.6% (Table 6.2). Measured nectar properties, however, did not differ among species with different pollination systems (volume, F1,2.76 = 6.82, P > 0.08; concentration, F1,2.84 = 0.32, P > 0.6).

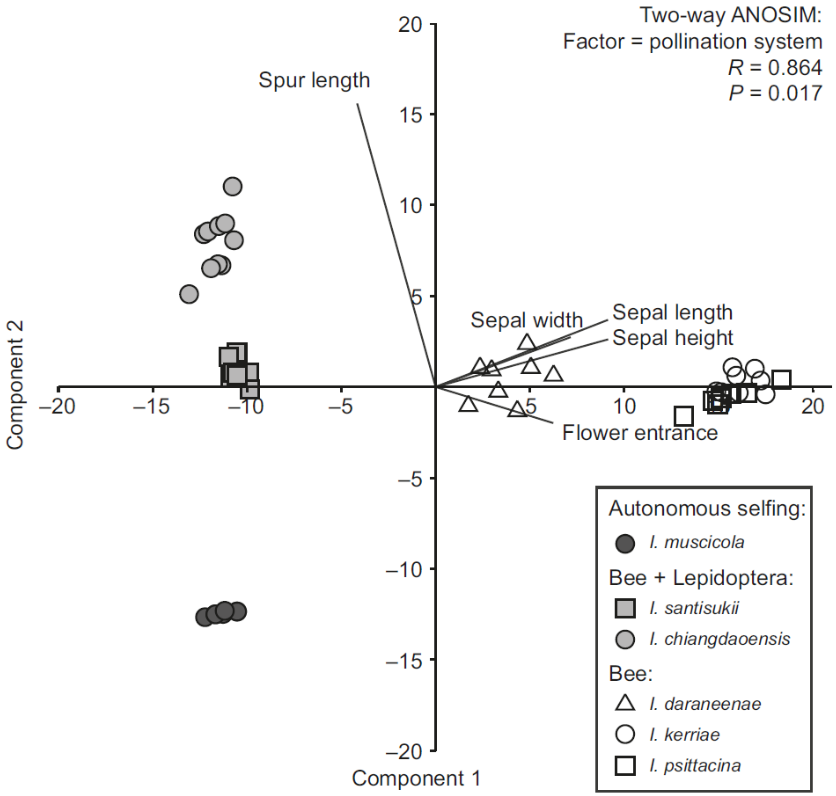

Fig. 6.6. First two axes of a principle components analysis of floral traits among six studied *Impatiens* spp. Lines indicate correlation of floral characters with the first two PCA axes. Species that share a pollination system are significantly more similar in floral traits compared with other species.

Table 6.2. Nectar volume and sugar concentration (mean ± SE) of *Impatiens* spp. with different pollination systems (Values between brackets indicate the number of species and total number of measurements, respectively.)

	Autogamous selfing (1, 5)	**Bee (3, 17)**	**Butterfly and bee (2, 14)**
Volume (lL)	0 ± 0	6.18 ± 1.83	13.55 ± 2.57
Concentration (% m/m)	n/a	38.14 ± 2.06	36.63 ± 2.97

6.5. Discussion

The results showed that six out of seven studied *Impatiens* spp. in the Chiang Dao Wildlife Sanctuary relied on animal visits for pollination. One autogamous species was characterized by small, nectarless flowers. Among the animal-pollinated species, two distinct pollination systems were identified. Two species were characterized by a bimodal pollination system involving bees and Lepidoptera. Flowers of these species were characterized by a relatively narrow floral entrance. Four species, characterized by flowers with a large floral chamber and short nectar spur, appeared specialized for bee pollination. A single bee species, Bombus haemorrhoidalis (Apidae) was a pollinator of five of the studied *Impatiens* spp.; differences in floral morphology, including floral asymmetry, enable pollen placement by bee-pollinated species on four distinct sites on the bee body. Our results therefore confirm that floral diversity among the studied *Impatiens* spp. is associated with different pollination systems, but also with divergent use of the same pollinator.

The association between pollination systems and floral traits detected in our study depends on correct identification of pollination systems. The accuracy of pollination system assignments might be compromised for two reasons. Firstly, observations were restricted to a single season and region for each species (e.g. Traveset & Saez, 1997; Price *et al*., 2005; De la Bandera & Traveset, 2006). As all species were studied in the same region and time period, however, we can exclude that spatial and/or temporal turnover in the pollination environment underlie the observed specialization in pollination systems. Therefore, the observed partitioning of the local pollinator fauna in our study area implies that the differences in specialized pollination systems are indeed associated with floral trait variation. Furthermore, several of the studied *Impatiens* spp. were characterized by a narrow distribution, including three species that are endemic to the study area. Spatial variation in pollination systems is unlikely to occur at the scale of a few kilometers (e.g. Johnson, 1997; Cosacov, Cocucci & Sersic, 2014; Van der Niet *et al*., 2014). Finally, by assigning functional pollinator groups rather than pollinator species to *Impatiens* spp., we allow for some turnover in pollinator species within functional groups without affecting the assignment of pollination systems. Conversely, definition of pollination systems on the basis of functional groups rather than pollinator species, may also lead to underestimation of the level of specialization of pollination systems, if, for example, members within each functional group do not behave and interact similarly with flowers. In our study we used broadly defined functional pollinator groups, such as bees, birds and Lepidoptera. There is ample evidence that variation among bee species in body size and in the ability to detect specific floral cues is associated with floral variation (e.g. Johnson, Steiner & Kaiser, 2005; Cosacov *et al*., 2014). In this context, it is notable that *I. daraneenae*, the only bee-pollinated species in our study that is not visited predominantly by *B. haemorrhoidalis*, occupies a distinct region in floral morphological space, which suggests that floral dimensions of this species differ from those of other bee-pollinated *Impatiens* spp. such as *I. kerriae* and *I. psittacina*. Further research is required to assess whether the functional groups used in our study adequately describe pollination systems in *Impatiens*, or whether further specialization for pollination by different species within functional groups may occur.

The second aspect of this study that could potentially affect accuracy of pollination system assignments is that, although we incorporated visitation frequency in the calculation of pollinator importance, we did not take visitation efficiency into account. The theoretical basis for expecting associations between pollinators and floral traits was articulated in the most effective pollinator principle (MEPP) (cf. Stebbins, 1970). According to this principle, flowers are moulded by visitors that visit plants most frequently and efficiently (but see Aigner, 2001). If pollination efficiency and frequency are not associated, a less frequent visitor may be a more important pollinator in terms of natural selection (e.g. Mayfield *et al*., 2001). The similarity in spur length of *I. santisukii* with that of bee-pollinated species might be explained, consistent with the MEPP, if bees are, as suggested by apparently much larger pollen loads, more efficient and thus more effective pollinators despite more frequent but probably relatively inefficient visitation by butterflies. Experiments designed to quantify the pollination efficiency, for instance by assessing fruit and seed set resulting from a single pollinator visit to a virgin flower (e.g. Janecek *et al*., 2011), could reveal important information on pollination efficiency of different functional pollinator groups. Such experiments would also provide insight into the role of the day-flying hawkmoth species M. bellis, which visited several *Impatiens* spp. studied here. Previous studies on *Impatiens* pollination also reported visits by Macroglossum spp. (Kato *et al*., 1991; Tian *et al*., 2004; Sreekala *et al*., 2008, 2011). The study by Sreekala *et al*. (2011) and Tian *et al*. (2004) mention that caught Macroglossum individuals carried *Impatiens* pollen. However, further studies are required to determine whether Macroglossum spp. are *Impatiens* pollinators or nectar robbers (e.g. Kishore *et al*., 2012). Our pollinator categorization is unlikely to be strongly affected by variation in pollination efficiency. Firstly, our analyses did not rely on relative efficiency of different functional pollinator groups and, secondly, we used the inclusive category ‘Lepidoptera’ to accommodate the potential role of Macroglossum as a pollinator.

Hypothesized associations between floral types and functional pollinator groups in *Impatiens* were limited to animal-pollinated categories (Vogel, 1954; Grey-Wilson, 1980). However, our study revealed that not all *Impatiens* spp. rely on animal visits for pollination and that the autogamous pollination system is associated with a distinct suite of floral traits. Initially, it was thought that *Impatiens* spp. were characterized by extreme herkogamy, the separation of male and female floral phases, by complete shedding of anthers before the stigma became receptive (Faegri & van der Pijl, 1979), which is often an efficient mechanism for avoiding autogamy and its negative consequences for fitness. However, other studies have already found evidence for autogamous pollination in *Impatiens* (Kato *et al.*, 1991; Tian *et al.*, 2004). In contrast with our findings, autogamy in Kato *et al.* (1991) and Tian *et al.* (2004) was associated with visits by pollinators and showy, large, nectar-producing flowers, perhaps serving as a mechanism of reproductive assurance (cf. Kalisz, Vogler & Hanley, 2004). Flowers of *I. muscicola* are small, do not produce nectar and appear adapted to self-pollination (cf. Tedder *et al.*, 2015), although we cannot completely exclude the possibility that the infrequent visits by ants and cockroaches contribute to fecundity in this species.

Among the animal-pollinated species, we identified two distinct floral syndromes, which were mainly determined by the shape of the lower sepal and length of the nectar spur. These two types of floral syndromes are largely congruent with the 'melittophilous' and 'psychophilous' types recognized by Vogel (1954) and the similar 'flat type' and a subcategory within the 'funnel type' flower of Grey-Wilson (1980). Pollination studies performed on African *Impatiens* spp. had already confirmed predicted associations between certain floral shapes and bird pollination (Janecek *et al.*, 2015). It appears that floral shape is a good indicator of broadly categorized pollination systems. Specifically, the size of the floral chamber varied between pollination systems, probably owing to different foraging behaviour of bees, which mostly crawl into the flower, and Lepidoptera which only insert their proboscis into the flower. More studies including pollinator observations are required to assess whether generalizations of associations between floral types and pollination systems are valid. Flower colour variation and nectar characteristics were not strongly associated with differences in pollination systems among species studied here. Although butterflies appeared to only visit pink-flowered *Impatiens* spp., bees and day-flying hawkmoths largely visited species regardless of flower colour, as functional pollinators and as nectar robbers. In the presumed absence of floral scent (none was detectable to the human nose), it seems likely that the main difference in floral traits associated with different functional pollinator groups involves traits related to the mechanical fit between flowers and visitors, rather than those related to pollinator attraction and behaviour (e.g. Miller, Raguso & Kay, 2014). If flowers do not filter visitors based on perceptional bias among animal species, flower visitation would be determined primarily by accessibility and palatability of the reward. Even visitors which are clearly mismatched with the flowers they visit, might occasionally successfully access the reward and pollinate flowers (e.g. de Merxem *et al*., 2009). In those cases, strong selection on floral traits might lead to rapid adaptation to the locally most effective pollinator. The species with bimodal pollination systems studied here might thus represent a transitional stage in a shift from one specialized pollination system to another (cf. Stebbins, 1970), rather than a stable state. Phylogenetic studies could reveal the signatures of frequent pollinator shifts by estimating the frequency of correlated shifts in floral traits and pollination systems throughout the evolutionary history of *Impatiens* (Van der Niet & Johnson, 2012).

Six out of the seven studied *Impatiens* spp. were visited and pollinated by bees; four species appear specialized for bee pollination. Several bee-pollinated species occur in the same area and partially coflower, raising the possibility of reproductive interference due to heterospecific pollen-transfer (HPT), which has been shown to reduce fecundity in other co-flowering *Impatiens* spp. that share pollinators (Tokuda *et al*., 2015). Among species studied here, HPT might be reduced through divergent use of the same pollinator (cf. Johnson, 2010). Specifically, four distinct sites of pollen placement on bee bodies were identified in our study (Fig. 6.5), partly owing to variation in the dimensions of the floral chamber, which might contribute to reduced HPT (cf. Waterman *et al*., 2011; Huang & Shi, 2013). Indeed, different pollen placement sites were previously suggested as mechanisms to avoid HPT in *Impatiens* (Kato *et al*., 1991; Bartos & Janecek, 2014). In *I. kerriae* and *I. daraneenae*, specific pollen placement was realized by floral asymmetry. The direction of asymmetry is monomorphic in populations for the species studied here, suggesting that, unlike enantiostyly, it probably does not serve as a mechanism for promoting outcrossing (e.g. Jesson & Barrett, 2002). Mechanisms of floral asymmetry that are not limited to the position of anthers and/or stigma or which do not only involve contortion of the corolla, are relatively rare in angiosperms (Endress, 2012), but were previously reported in *Impatiens*, also as a supposed mechanism to avoid reproductive interference (Kato *et al*., 1991). Further *Impatiens* spp. in Southeast Asia also appear to be characterized by asymmetrical flowers (Yu, 2012), suggesting that floral asymmetry might be relatively widespread in this region. Three alternative processes related to avoidance of HPT could generate a large number of species with asymmetrical flowers. First, the trait might provide a strong ecological advantage for invading existing *Impatiens* communities through the avoidance of competition ('community invasion' hypothesis, Sargent & Ackerly, 2008). As a result, clades with asymmetrical flowers might become widespread and proliferate. A signature of this process would be enhanced diversification rates of clades with asymmetrical flowers. Second, selection might favour the evolution of asymmetrical flowers as a mechanism of character displacement to avoid HPT ('character displacement' hypothesis, Armbruster & Muchhala, 2009). This would be expressed as frequent shifts towards floral asymmetry and the occurrence of intraspecific polymorphism for asymmetry (i.e. symmetrical and asymmetrical ecotypes), depending on the local *Impatiens* community. Finally, floral asymmetry might serve as a mechanism of reproductive isolation through reinforcement (the Pedicularis L. type of ethological isolation in the 'reproductive isolation' hypothesis; Grant, 1994). This would lead to similar patterns to those as predicted for the character displacement hypothesis, but would specifically require that communities are composed of closely related species capable of hybridizing. Given that the reproductive isolation hypothesis was rejected in a similar study system due to insufficiently precise pollen placement (Armbruster *et al*., 2014) and since bee-pollinated species with different pollen placement sites are not sister species (Ruchisansakun *et al*., 2015), it is unlikely that the reproductive isolation hypothesis explains the common occurrence of asymmetry in *Impatiens*. Phylogenetic evidence would be invaluable to distinguish between the 'community invasion' and 'character displacement' hypotheses (Sargent & Ackerly, 2008; Eaton *et al*., 2012). Although no formal analyses have been performed, the phylogenetic tree of Ruchisansakun *et al*. (2015) suggests that floral asymmetry has evolved multiple times, a hypothesis that is further supported by the distinct and therefore probably nonhomologous mechanisms of floral asymmetry in *I. kerriae* and *I. daraneenae*.

Our study confirms predictions about associations between floral traits and pollination systems for one of the most spectacular floral radiations of the Old World tropics. Future work should focus on inclusion of more taxa and floral types, in particular, those hypothesized to be associated with long-tongued hawkmoth pollination (Vogel, 1954; Grey-Wilson, 1980), which are as yet unstudied. However, to clarify the role of pollinators in driving floral divergence, it is also important that evolutionary patterns are investigated, by implementing phylogenetic methods, and to evaluate the processes that might have generated these patterns. In particular, the role of pollinators as selective agents on floral traits could be tested by using manipulative experiments.

ACKNOWLEDGEMENTS

We thank Mr Somsak Thithichayaporn, the chief of Chiang Dao Wildlife Sanctuary, for permission to work inside the reserve. Adam Shuttleworth helped with statistical analyses, Maarten van ’t Zelfde assisted with the design of the distribution map. We thank Dr Karl Duffy and two anonymous reviewers for useful comments on an earlier draft of this paper. This research was funded by a Development and Promotion of Science and Technology talents project (DPST) scholarship, provided by the Thai Institute for the Promotion of Teaching Science and Technology (IPST).