Pollination-precision hypothesis: support from native honey bees and nectar bats

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Summary

• The evolution of floral traits is often considered to reflect selection for increased pollination efficiency. Known as the pollination-precision hypothesis, increased pollination efficiency is achieved by enhancing pollen deposition on precise areas of the pollinator. Most research to date addressing this hypothesis has examined plant species that are *a priori* predicted to place pollen precisely, but we still lack comparisons with species predicted to have low pollination efficiency.

• We studied 39 plant species with diverse floral morphologies and measured the precision of pollen placement on two pollinator groups: honey bees (genus *Apis*) and nectar bats (family Pteropodidae). Pollen was collected from four locations of each pollinator's body (bees: dorsal thorax, ventral thorax, dorsal abdomen, ventral abdomen; bats: crown, face, chest, wing) to calculate pollen placement precision using Pielou's evenness index. We also quantified variation in floral design by scoring floral symmetry, corolla fusion, floral orientation and stamen number.

• We confirm the importance of four floral character states (bilateral symmetry, fused corollas, horizontal orientation and reduced stamen number) in promoting precise pollen placement on diverse pollinators.

• Our findings provide phylogenetically corrected, empirical support that the evolution of the four floral characters reflect selection for enhanced precision of pollen placed on pollinators.

Introduction

Floral traits that increase pollination efficiency through precise pollen placement on pollinators (also referred to sometimes as 'floral specialisation', *sensu* Armbruster & Muchhala, 2009), are considered a hallmark of angiosperm evolution (Darwin, 1862; Fenster *et al.*, 2004; Waser & Ollerton, 2006; Armbruster, 2014, 2017), reflected in long-term trends of floral trait evolution (Bessey, 1915). Increasing evidence supports the notion that the evolution of precise pollen placement is an outcome of pollinator-mediated selection (Grant, 1949; Faegri & van der Pijl, 1966; Stebbins, 1970; Fenster *et al.*, 2004; Citerne *et al.*, 2010; van der Niet & Johnson, 2012; Rosas-Guerrero *et al.*, 2014) associated with increased conspecific pollen transfer and reduced pollen loss (Inouye *et al.*, 1994; Neal *et al.*, 1998; Nikkeshi *et al.*, 2015; Culbert & Forrest, 2016). This hypothesis has been given several names, including the 'pollen position hypothesis' (Neal *et al.*, 1998), 'pollination-precision hypothesis' (Fenster *et al.*, 2009; Nikkeshi *et al.*, 2015) and 'pollen-placement-accuracy hypothesis' (Culbert & Forrest, 2016), the

last of which also considers 'bias' (i.e. the optimal position of pollen placement and stigma contact) in addition to 'precision' (i.e. consistent pollen placement or stigma contact) (*sensu* Hansen *et al.*, 2006; Armbruster *et al.*, 2009). The evolution of increasing pollination precision has implications across numerous levels of biological organisation, from the community level structure of plant–pollinator interactions (Lázaro *et al.*, 2008) to patterns of diversification (O'Meara *et al.*, 2016).

The precision of pollen placement on pollinators is influenced by numerous floral traits, resulting in a continuum from low to high precision. Floral traits predicted to influence the precision of pollen placement on pollinators include floral symmetry, corolla fusion, floral orientation and stamen number (Stebbins, 1951; Fenster *et al.*, 2009; O'Meara *et al.*, 2016). Floral symmetry, corolla fusion and floral orientation are all expected to influence pollination precision by constraining how pollinators approach flowers (Stebbins, 1951; Herrera *et al.*, 2008; Fenster *et al.*, 2009; Culbert & Forrest, 2016; O'Meara *et al.*, 2016; Yu *et al.*, 2021). Therefore, we expect more consistent pollen placement on pollinators by zygomorphic (bilaterally symmetric) flowers compared with actinomorphic (radially symmetric) flowers (Culbert & Forrest, 2016), by sympetalous flowers (with fused corollas) compared with

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polypetalous flowers (with unfused corollas) (Herrera *et al.*, 2008) and by horizontally oriented (or semipendant) flowers compared with vertically oriented flowers (Fenster *et al.*, 2009; Yu *et al.*, 2021). Additionally, flowers with numerous stamens are expected to have more opportunities to contact different areas of a pollinator's body (i.e. less precise pollen placement) compared with flowers with few stamens (Stebbins, 1951; O'Meara *et al.*, 2016). Therefore, zygomorphy, sympetaly, horizontal or semipendant orientation and reduced stamen number are expected to confer a fitness advantage through increased conspecific pollen transfer and reduced pollen loss.

Whereas the pollination-precision hypothesis has been cited numerous times as an underlying factor for floral evolution (Walker-Larsen & Harder, 2000; Sargent, 2004; Herrera *et al.*, 2008; Muchhala *et al.*, 2010; Specht *et al.*, 2012; O'Meara *et al.*, 2016; Reyes *et al.*, 2016), only a handful of studies have provided empirical support that floral form is directly related to pollination precision (e.g. Muchhala & Potts, 2007; Muchhala, 2007; Armbruster *et al.*, 2014). Other studies, using indirect evidence, have demonstrated that zygomorphic flowers exhibit less variation in floral size compared with actinomorphic flowers, with the implication that more consistent flower size leads to more consistent pollen placement on pollinators (Wolfe & Krstolic, 1999; Ushimaru *et al.*, 2007; Herrera *et al.*, 2008; Gong & Huang, 2009; Lázaro & Totland, 2014; Nikkeshi *et al.*, 2015; Diller, 2016). Additionally, floral traits involved in pollen placement (e.g. stamen and style length) show less variance and greater integration than traits involved in pollinator attraction (e.g. corolla diameter) (Rosas-Guerrero *et al.*, 2010). Furthermore, prior studies have shown that floral orientation and floral symmetry influence pollinator approach to the flower, influencing how precisely stamens contact pollinators (Fenster *et al.*, 2009; Culbert & Forrest, 2016; Yu *et al.*, 2021). These studies provide support for the pollination-precision hypothesis, but we still lack empirical studies that compare a broad range of floral morphologies to assess which floral traits contribute to precise pollen placement across diverse groups of pollinators.

In this study, we empirically tested which floral character states confer precise pollen placement on the pollinator's body using data from two pollinator groups that are native to the study areas: ecologically generalist honey bees (genus *Apis*) and ecologically specialised nectar bats (family Pteropodidae). We quantified the floral traits that Stebbins (1951) and O'Meara *et al.* (2016) predicted should impact pollen placement on the pollinator – floral symmetry, corolla fusion and the ratio of stamens to perianth parts – as well as floral orientation (Fenster *et al.*, 2009). We hypothesised that flowers exhibiting bilateral symmetry, fused corollas, horizontal or semipendant orientation and reduced stamen number would place pollen more precisely on the pollinator than flowers exhibiting the alternative character states of radial symmetry, unfused corollas, vertical orientation and relatively greater stamen number. We therefore collected pollen from the bodies of bat and bee pollinators foraging at plants with diverse floral morphologies (six plant taxa visited by bats, 33 plant species visited by bees) and we consider our results using phylogenetic comparative approaches.

Materials and Methods

Plant study species

For our bat-plant data set, we focused on the six most common plant taxa found in the diets of nectarivorous bats in Thailand: *Ceiba pentandra* (L.) Gaertn., *Durio zibethinus* L., *Musa acuminata* Colla, *Oroxylum indicum* (L.) Kurz, *Parkia* spp. and *Sonneratia* spp. (Stewart & Dudash, 2017b) (Supporting Information Table S1). For our bee-plant data set, we sampled all plant species that we observed at least three *Apis* bees foraging at (found carrying at least four pollen grains), resulting in 33 plant species (32 genera, 22 families; Table S2). When counting pollen collected from bat and bee floral visitors to quantify pollen placement precision, we only quantified pollen grains from these plant study species.

Quantifying pollen placement on bats

We used pollen placement data collected from 1058 wild foraging bats (Fig. 1c,d) that were netted in southern Thailand (Phatthalung, Satun, Songkla and Trang provinces) between March 2013 through August 2014. The region is dominated by a mixed agriculture–forest landscape and nectarivorous bats and bat-pollinated plants are common (Stewart *et al.*, 2014). Bats were uniquely marked and we only used pollen data collected from the first capture. Most data were obtained from *Eonycteris spelaea* bats (723 individuals), but we also included data from all other bat species netted (*Macroglossus minimus*, 114 individuals; *M. sobrinus*, 77 individuals; *Rousettus leschenaulti*, 52 individuals; *R. amplexicaudatus*, one individual; *Cynopterus horsfieldii*, 40 individuals; *C. sphinx*, 24 individuals; *C. brachyotis*, 23 individuals; *Megaerops ecaudatus*, four individuals). The *Eonycteris* and *Macroglossus* species are specialised nectar-feeding bats, while the other species consume both fruit and floral resources (Stewart *et al.*, 2014). Data were pooled across all bat species given the small sample sizes for most species (i.e. few individuals per bat species and/or few plant species visited per bat species). The methods for netting bats and collecting pollen are described in Stewart & Dudash (2017a). Briefly, mist nets were placed in the canopy in front of open, unmanipulated flowers of our plant study species between 18:00 to 24:00 h, and netted bats were swabbed with fuchsin glycerin gelatin to collect pollen from four areas of the body: the top of the head (crown), face, chest and ventral side of one wing. Samples from each area of the body were mounted on separate slides and pollen grains were identified and counted using a compound light microscope.

We also used pollen placement data collected from 85 wild bats used in flight cage experiments conducted in southern Thailand between February 2014 through January 2015. Most data were obtained from *E. spelaea* (72 bats), followed by *R. leschenaulti* (eight bats), *M. sobrinus* (four bats) and *M. minimus* (one bat). The methods for the flight cage experiments and pollen collection are described in Stewart & Dudash (2016). Briefly, each wild bat was placed in a flight cage with virgin flowers of our plant study species. After bats foraged on the study flowers,

Fig. 1 Photographs of (a) *Apis florea* visiting a *Portulaca grandiflora* flower, (b) *Apis cerana* visiting a *Ruellia simplex* flower, (c) *Eonycteris spelaea* visiting *Durio zibethinus* flowers and (d) *Eonycteris spelaea* visiting an *Oroxylum indicum* flower. Flowers in panels (a) and (c) have radial symmetry, unfused corollas, vertical (or varied) orientation and numerous stamens. Flowers in panels (b) and (d) have bilateral symmetry, fused corollas, horizontal or semipendant orientation and fewer stamens. (Bee photographs taken by Alyssa Stewart, bat photographs taken by Merlin Tuttle.)

pollen was collected from the same four areas of the body, mounted on slides and counted as explained above.

Quantifying pollen placement on bees

We used pollen placement data collected from 180 wild *Apis* bees (Fig. 1a,b) that were caught between April through September 2018 in central Thailand (Bangkok and Nakhon Pathom province). The area is mainly comprised of urban, suburban and agricultural habitats, with both naturally occurring and cultivated vegetation. We focused on the three most common native *Apis* species found in Thailand: *A. cerana*, *A. dorsata* and *A. florea*. Data were pooled for all bee species to maximise the number of plant species analysed and because the three species are similar in size relative to the flowers they visit. *Apis dorsata* is the largest of the three (intertegular distance, ITD: 3.9 mm), followed by *A. cerana* (ITD: 3.0 mm) and then *A. florea* (ITD: 2.2 mm) (Tangtorwongsakul *et al.*, 2018). For size reference, the average ITD of *A. mellifera* can range between 2.93 and 3.07 mm (Kolmes & Sam, 1991; Santos & Serrão, 2006). All three *Apis* species are polylectic and forage on diverse plant species (Suwannapong *et al.*, 2013; Stewart *et al.*, 2018). While it is possible that some of the bee-visited plant species are not actually pollinated by bees, we were still interested in the precision of pollen placement on bee visitors, since bilateral symmetry, corolla fusion and horizontal orientation are expected to constrain the movement of all floral visitors to some extent, not just actual pollinators, and flowers with fewer stamens are expected to have fewer opportunities to contact multiple areas of a floral visitor's body.

For each plant species that we observed *Apis* species foraging at, we collected 3–5 bees per *Apis* species. Bees were collected using plastic Ziploc bags; after a bee entered an open, unmanipulated flower, the bag was placed over the flower, therefore trapping the bee when it flew out. Bees were cold immobilised following Frost *et al.* (2011); specifically, each bagged bee was placed in a cooler with ice for 1–3 min, until the bee was motionless. Once the bee was cold immobilised, pollen was collected from four areas of the body: ventral thorax, dorsal thorax, ventral abdomen and dorsal abdomen. Pollen was collected using fuchsin glycerin gelatin in a modified syringe, which allowed us to dab the gel on each specific area of the pollinator's body, as described in Stewart & Dudash (2016). Once pollen collection was complete, the bee was marked on the abdomen with a permanent marker (to prevent resampling of the same individual), placed in the sun and allowed to recover; all individuals were observed to recover and fly away. The pollen samples from each area of the body were mounted on separate slides and a reference slide was also created using pollen collected directly from the flower. When examining pollen samples via compound light microscope, only pollen grains of the focal plant species (i.e. the plant species at which the bee was collected) were counted. Individuals that carried fewer than four pollen grains were excluded from the analyses, resulting in 180 individuals total (105 *A. cerana*, 28 *A. dorsata* and 47 *A. florea*). We chose four pollen grains as the cut-off point because individuals carrying fewer than four pollen grains are mathematically unable to achieve a pollen placement precision value of zero (i.e. they are biased towards greater precision) based on our calculation of pollen placement precision (please refer to the section on Statistical analyses).

Quantifying floral traits

Each plant study species was scored for the following floral traits that were predicted to affect the precision of pollen placement: floral symmetry, corolla fusion, floral orientation and ratio of stamens to perianth parts (Fig. 1). We had originally planned to include corolla presence as well (following Stebbins, 1951; O'Meara *et al.*, 2016), however, all plant study species had corollas, so this trait was not included in the analyses. Scores for each trait ranged from 0 (indicating a character state predicted to place

pollen imprecisely) to 1 (indicating a character state predicted to place pollen precisely). Categories for each floral trait were decided *a priori* to facilitate quick and accurate scoring. In the following paragraphs we describe quantification of each of the four floral traits in greater detail.

Floral symmetry: plants were assigned a 0 if their flowers exhibited radial symmetry, 1 if they exhibited bilateral symmetry, or 0.5 if the species had both radial and bilateral flowers (e.g. *Coriandrum sativum*, Apiaceae). Among our study species, stamen symmetry always corresponded with corolla symmetry (i.e. species with zygomorphic corollas always had zygomorphic stamens, and species with actinomorphic corollas always had actinomorphic stamens), therefore, our use of the term 'floral symmetry' includes both corolla symmetry and stamen symmetry.

Floral orientation: plants were assigned a 1 if their flowers were consistently oriented horizontally or semipendant (the face or entrance to the flower perpendicular to the ground or angled within 45° of perpendicular to the ground, following Fenster *et al.*, 2009); plants were assigned a 0 if their flowers were oriented vertically (the face or entrance to the flower open towards the sky) or in multiple directions (e.g. some flowers oriented vertically and some oriented horizontally or semipendant). Species with vertical and mixed orientation were grouped together as species with vertical orientation often had bent or tilted stems (resulting in flowers with mixed orientation) and plants spanned a continuum ranging from species with mostly vertically oriented flowers to species with truly mixed floral orientation (i.e. flowers oriented in numerous directions in relatively equal proportions).

Corolla fusion: plants were assigned a 0 if their flowers had free petals or tepals, 0.5 if there was 1–50% fusion (relative to petal or tepal length), or 1 if there was 51–100% fusion (relative to petal or tepal length).

Ratio of stamens to perianth parts (from this point forwards, S : P ratio): plants were assigned a 0 if the ratio was greater than one (more stamens than perianth parts), 0.5 if the ratio was equal to one (equal numbers of stamens and perianth parts), or 1 if the ratio was less than one (fewer stamens than perianth parts).

When quantifying the floral traits of our plant study species, we examined flowers from at least three plants per species. The floral traits of all six bat-plant taxa and all 33 bee-plant taxa were scored the same way. Some of our plant study species bore individual flowers, while others bore inflorescences; for consistency, we scored all plant species at the level of the individual flower or floret. It is true that, for some plant species, the entire inflorescence may be the unit under selection for increased precision, rather than the individual florets, such as when the pollinator perceives the entire inflorescence as the unit of interest and interacts with multiple florets simultaneously. However, we did not want to introduce bias by arbitrarily scoring some species at the level of the inflorescence and some species at the level of the floret based on how we perceived the floral units, which may be different than how the pollinators perceive them. It is also possible that, for a given plant species, some of its pollinators may impose selection on the entire inflorescence while other pollinators impose selection on the individual florets. We therefore decided that the most

objective, and conservative, method was to score all plant species at the level of the individual flower or floret. We also removed all pseudanthial species from the data sets (four bee-plant species and one bat-plant species) and re-ran the analyses to confirm that the effect of floral design on pollen placement precision was not being driven by the pseudanthial arrangement of florets (please refer to Notes S1).

Statistical analyses

To calculate the precision of pollen placement on pollinators, we adapted the traditional measure of species evenness, Pielou's evenness index, which is calculated as H' (Shannon's diversity index) divided by $H'_{\max}$ (the maximum possible value of H'). However, instead of calculating H' from the proportion of each species in a community, we calculated it from the proportion of pollen found on each area of the pollinator's body. In other words, for the equation $H' = -\sum_{i=1}^{S} p_i \log_e p_i$, values of p_i were calculated from the number of pollen grains on body part i divided by the total number of pollen grains. As we collected pollen from four areas of the pollinator, $H'_{\max}$ was always equal to $\log_e(4)$. Dividing H' by $H'_{\max}$ resulted in values of pollen placement evenness ranging from zero (less evenness) to one (more evenness). We then subtracted these evenness values from one to obtain pollen placement precision. Therefore, our final equation for pollen placement precision was $1 - (H'/H'_{\max})$, where values closer to zero indicated less precision (i.e. pollen was evenly distributed across all areas of the pollinator's body) and values closer to one indicated more precision (i.e. pollen was found on some areas of the body more than others).

For all subsequent procedures, we analysed the bat and bee data separately. Because we knew the four floral traits were inter-correlated (Fig. S1), we performed partial least squares regression (PLS) with pollen placement precision as the response variable and the four floral traits as explanatory variables using the MDA-TOOLS package in R (Kucheryavskiy, 2020). We chose PLS as it is a dimension-reduction method that incorporates the correlation between response and explanatory variables when orienting the latent variables (Maitra & Yan, 2008). The optimal number of components was selected using root-mean-square error (RMSE) values and a single PLS component was found to be optimal for both the bat and bee data sets. The significance of each explanatory variable (i.e. each floral trait) on the first PLS component was determined by jack-knifing the regression coefficients (Kucheryavskiy, 2020).

We then tested whether the PLS component significantly influenced pollen placement precision using phylogenetic generalised least squares regression (PGLS), which allowed us to examine patterns while incorporating correlations introduced by a shared phylogenetic history among the plant study species (package NLME; Pinheiro *et al.*, 2021). A separate phylogeny was constructed for the bat-plants (Fig. 2a) and bee-plants (Fig. 2b) using the V.PHYLOMAKER package (Jin & Qian, 2019) and the phylogenetic correlation matrix for each tree was computed using the APE package (Paradis & Schliep, 2019). Separate PGLS analyses were performed on the bat and bee data sets; for each analysis,
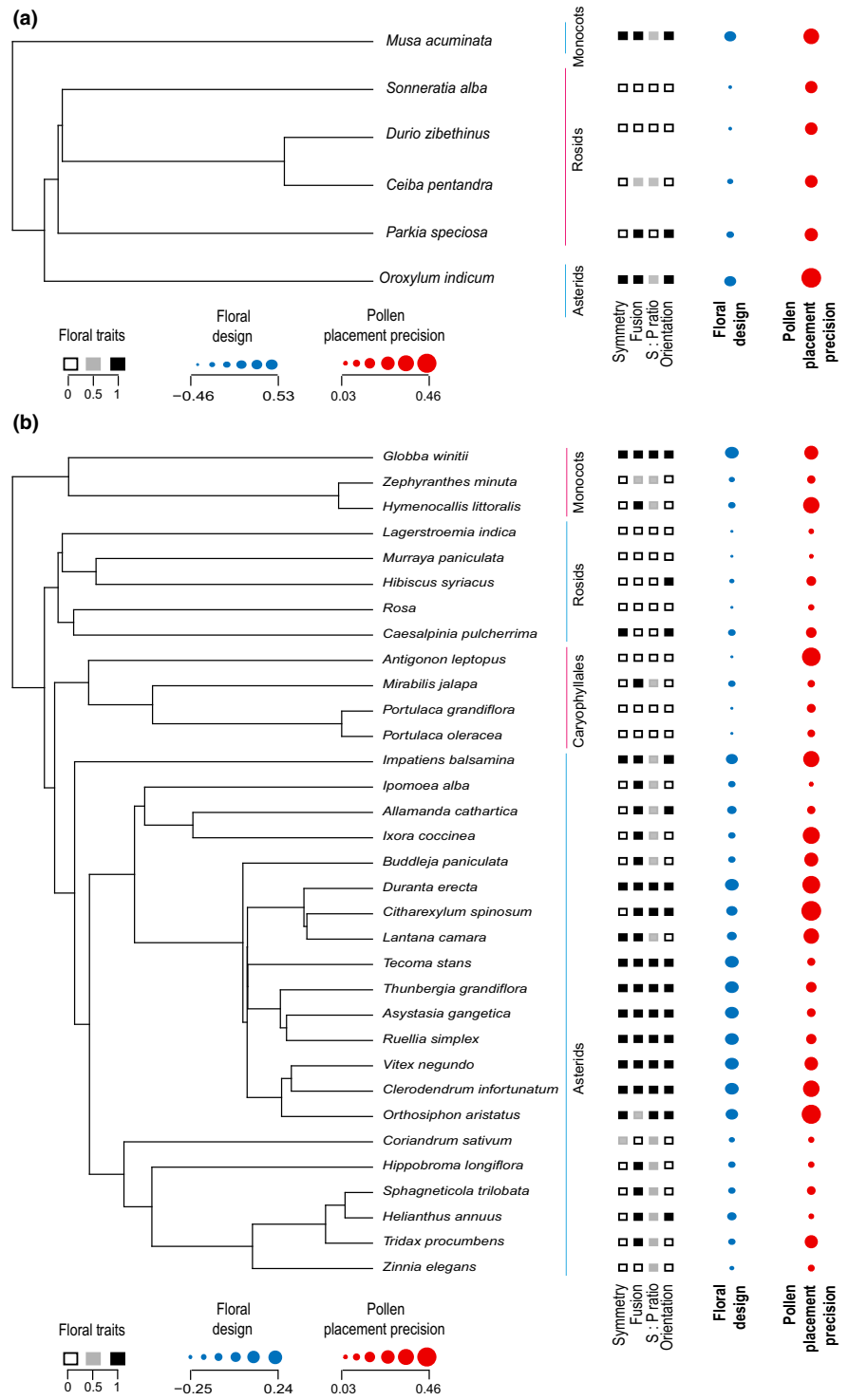
(a)

(b)

Fig. 2 Phylogenetic trees of the plant study species visited by (a) nectar bats and (b) honey bees. For each plant species, values for floral traits (squares), floral design (blue circles) and pollen placement precision (red circles) are shown on the right. For the floral traits (floral symmetry, corolla fusion, ratio of stamens to perianth parts and floral orientation), 0 (white squares) represents the character state predicted to place pollen imprecisely on the pollinator, 1 (black squares) represents the character state predicted to place pollen precisely and 0.5 (grey squares) represents intermediate states (please refer to main text for details). Floral design is a latent variable comprised of the four floral traits; more positive numbers (larger circles) are associated with bilateral symmetry, fused corollas, horizontal or semipendant orientation and reduced stamen number, while more negative numbers (smaller circles) are associated with radial symmetry, unfused corollas, vertical orientation and numerous stamens. Therefore, species with four black squares (in the floral trait columns) have the largest blue circles in the Floral Design column, while species with four white squares have the smallest blue circles in the Floral Design column. Pollen placement precision is a measure of how precisely pollen is placed on the pollinator's body, with larger numbers (larger circles) representing greater precision.

pollen placement precision was the response variable, the first PLS component was the explanatory variable and the phylogenetic correlation matrix was included to incorporate the phylogenetic signal among plant species. The significance of the explanatory variable was assessed with likelihood ratio testing and considered significant when $P < 0.05$. We confirmed our results with permutation testing using the package PREDICTMEANS (Luo

et al., 2021); the full PGLS model for each data set was analysed with 9999 permutations. Additionally, we estimated the phylogenetic signal (λ) using the PHYLOLM package (Ho & Ane, 2014) and examined variance in the response variable explained by the explanatory variable and the phylogeny using the RR2 package (Ives & Li, 2018). All analyses were performed in R 4.0.2 (R Core Team, 2020).

Results

Average values of pollen placement precision were higher and less variable for bat-plants (median = 0.273, IQR = 0.071; Figs 2a, 3a) than those of bee-plants (median = 0.182, IQR = 0.227; Figs 2b, 3b). For the bat data set, all four floral traits contributed significantly to the first PLS component (symmetry: regression coefficient = 0.306, $P = 0.027$; fusion: coeff. = 0.227, $P = 0.005$; S : P ratio: coeff. = 0.214, $P = 0.018$; orientation: coeff. = 0.241, $P = 0.009$; Fig. 4a). For the bee data set, two floral traits were significant (symmetry: coeff. = 0.134, $P = 0.038$; S : P ratio: coeff. = 0.151, $P = 0.021$) and two floral traits were marginally significant (fusion: coeff. = 0.127, $P = 0.059$; orientation: coeff. = 0.115, $P = 0.067$; Fig. 4b). Among the bat data, the first PLS component explained 73.9% of the variation in the explanatory variables (floral traits) and 72.7% of the variation in the response variable (pollen placement precision). Among the bee data, the first PLS component explained 66.2% of the variation in the explanatory variables (floral traits) and 18.5% of the variation in the response variable (pollen placement precision). For both data sets, the positive regression coefficients of all four floral traits revealed that they contributed to the first PLS component in the same direction

(i.e. more positive values on the PLS component were associated with the character states predicted to place pollen precisely: zygomorphy, fused corollas, horizontal or semipendant orientation and reduced stamen number).

Additionally, the PGLS analyses revealed that the first PLS component (a latent variable describing floral design with respect to pollen placement on pollinators) had a significant positive effect on pollen placement precision for both the bat data set ($\chi_1^2 = 4.97$, $P = 0.026$; Fig. 5a) and the bee data set ($\chi_1^2 = 5.34$, $P = 0.021$; Fig. 5b). The permutation test results were also significant for both the bat PGLS model ($F = 10.63$, df = 1, $P = 0.007$) and the bee PGLS model ($F = 7.04$, df = 1, $P = 0.013$). Among plant species visited by bees, the phylogenetic signal was moderately high for pollen placement precision ($\lambda = 0.54$) and very high for the first PLS component (i.e. floral design with respect to pollen placement on pollinators; $\lambda = 1$). Partitioning variances in the bee PGLS model revealed that the first PLS component explained 18.5% of the variance in pollen placement precision and phylogeny explained 0% since all of the phylogenetic signal in pollen placement precision was already explained by the first PLS component. As calculation of lambda requires a sample size of at least 30 (Kamilar & Cooper, 2013), we did not test the phylogenetic signal for bat-plants ($n = 6$ plant taxa).
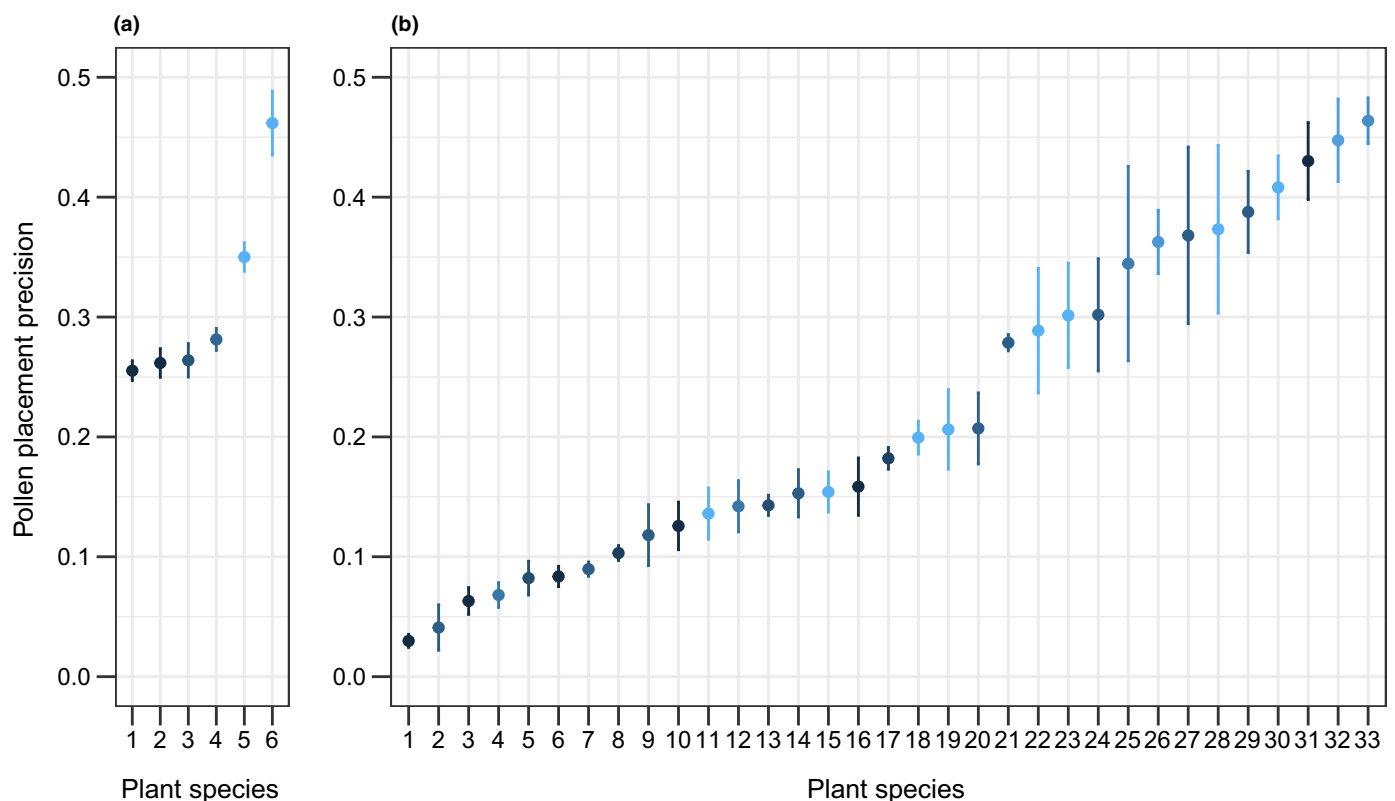

Fig. 3 Average precision of pollen placement on pollinators (mean $\pm$ 1SE) for plant species visited by (a) nectar bats and (b) honey bees. The colour of each point indicates floral design (as measured by PLS component 1; please refer to text for details); along this colour gradient, darker colours represent floral morphologies associated with actinomorphy, unfused corollas, vertical orientation and numerous stamens, while lighter colours represent floral morphologies associated with the alternative character states of zygomorphy, fused corollas, horizontal or semipendant orientation and few stamens. (Please refer to Supporting Information Table S1 for the names of plant species visited by bats and Table S2 for the names of plant species visited by bees.)
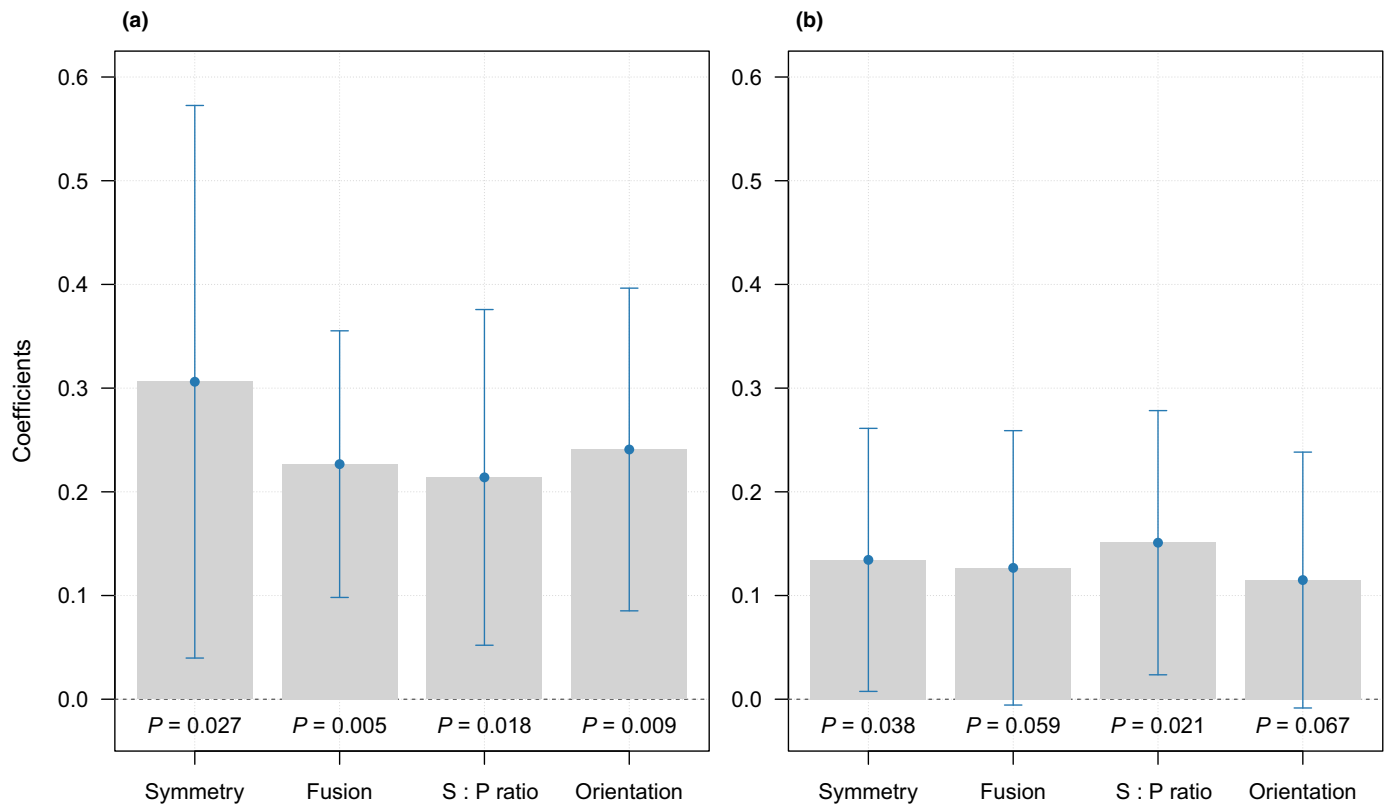
Fig. 4 Partial least squares (PLS) regression coefficients and jackknife 95% confidence intervals of four floral traits (floral symmetry, corolla fusion, ratio of stamens to perianth parts (S : P ratio) and floral orientation) contributing to PLS component 1 for plant species visited by (a) nectar bats and (b) honey bees. Floral traits with confidence intervals not overlapping zero ($P < 0.05$) contribute significantly to PLS component 1, which indicates zygomorphy, fused corollas, horizontal orientation and reduced stamen number in one direction, and actinomorphy, unfused corollas, vertical orientation and numerous stamens in the opposite direction. PLS component 1 explained 73.9% of the floral trait variation in plant species visited by bats and 66.2% of the floral trait variation in plant species visited by bees.

Discussion

We quantified pollen placement on nine species of nectar bats and three species of honey bees native to Thailand following floral visits to six bat-visited plant taxa and 33 bee-visited plant taxa. We found consistent evidence that floral character states long hypothesised to restrict pollinator movement and guide the pollinator's interaction with the flower's reproductive parts do result in more precise pollen placement on the pollinator's body in both pollinator groups. In the following sections we discuss the evolutionary and ecological consequences of the evolution of floral character states related to increased pollen placement precision.

Contribution of floral traits towards precise pollen placement on pollinators

Our study demonstrates that all four floral traits contributed towards variation in precise pollen placement on pollinators. We found that the first PLS component indicates zygomorphy, fused corollas, horizontal or semipendant orientation and reduced stamen number in one direction, and actinomorphy, unfused corollas, vertical orientation and numerous stamens in the opposite

direction. Therefore, the first PLS component is a measure of overall floral design and a proxy for how precisely a plant species places pollen on pollinators. Similar to our findings, Stebbins (1951) reported positive correlations between zygomorphy, fused corollas and reduced stamen number. Stebbins (1951) did not include floral orientation in his analysis, but our results showed that horizontal or semipendant orientation is another floral state associated with precise pollen placement on pollinators, as proposed by Fenster *et al.* (2009). Moreover, our PLS results revealed that all four floral traits contributed relatively equally to precise pollen placement on pollinators, with each trait explaining between 21–31% of the variation among bat-plants and between 11–15% of the variation among bee-plants. Because PLS attempts to explain variation among the independent variables while maximising their correlation with the dependent variable (i.e. pollen placement precision), our results indicate that all four floral traits are similarly important in explaining variation among animal-pollinated plants with respect to the precision of pollen placement on pollinators.

Effect of floral design on pollen placement precision

As predicted, floral design had a significant positive effect on pollen placement precision for both bat-plants and bee-plants. The
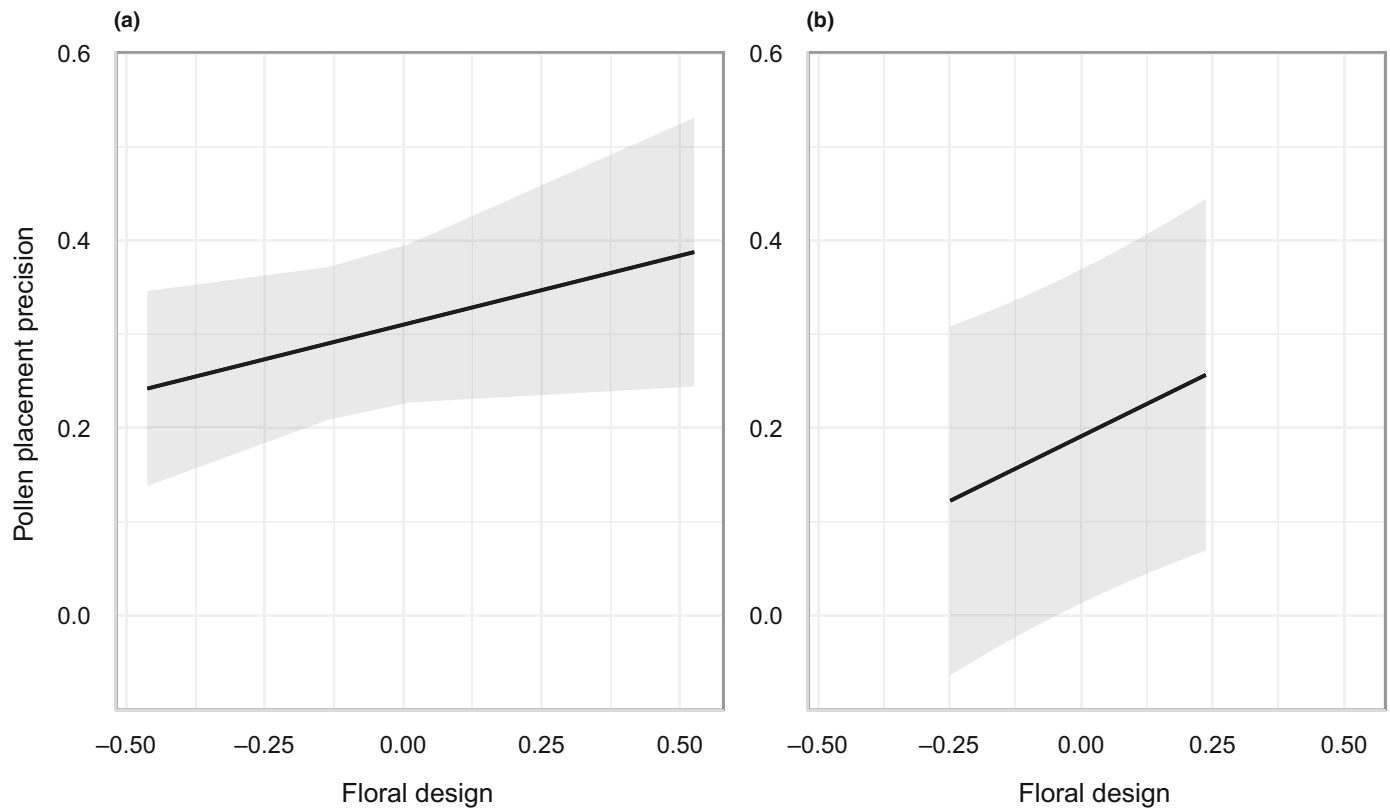
Fig. 5 Phylogenetic generalised least squares (PGLS) regression results with 95% confidence intervals (shaded regions) for plant species visited by (a) nectar bats and (b) honey bees. Floral design (as measured by PLS component 1; please refer to text for details) significantly influences the precision of pollen placement on pollinators for both plant species visited by bats ($\chi^2_1 = 4.97$, $P = 0.026$) and plant species visited by bees ($\chi^2_1 = 5.34$, $P = 0.021$). More positive values of floral design are associated with bilateral symmetry, fused corollas, horizontal or semipendant orientation and reduced stamen number, while more negative values are associated with radial symmetry, unfused corollas, vertical orientation and numerous stamens.

floral character states of zygomorphy, fused petals, horizontal or semipendant orientation and reduced stamen number have long been assumed to improve intraspecific pollen transfer by improving the precision of pollen placement on the pollinator's body (Inouye *et al.*, 1994; Neal *et al.*, 1998; Nikkeshi *et al.*, 2015; Culbert & Forrest, 2016) and our study empirically confirmed that these hypotheses were true. Numerous studies have examined plant species that are able to achieve precise pollen placement on bees (Armbruster *et al.*, 2014; Huang *et al.*, 2015; Ruchisansakun *et al.*, 2016; van der Niet *et al.*, 2020) and bats (Muchhala & Potts, 2007; Muchhala, 2007; Maguiña & Amanzo, 2016). However, studies examining plant species that place pollen broadly on pollinators are less common (but please refer to Lemke, 1985; Butler & Johnson, 2020), precluding comparison across diverse floral designs until now.

While the four floral character states positively influenced the precision of pollen placement on both bats and bees, precision values were higher and less variable overall on bats than bees. This difference may be because nectarivorous bats are proportionally larger relative to the flowers they forage on, so pollen is placed over a relatively smaller area, resulting in higher precision. By contrast, honey bees are generally smaller than the flowers they forage on, presumably making it more difficult for pollen to be placed precisely on such a small target. A second possible

explanation is that almost all of our bat-pollinated plant study species are native to the study area (five out of six species; Table S1) and presumably share a close evolutionary history with the native bat pollinators. By contrast, for the bee data set, while all three honey bee species are native to Thailand, most of the plant species studied are exotic (19 out of 30 species for which we have distribution information; Table S2) and may be pollinated by other pollinator groups in their native range. It is therefore notable that we still observed a significant correlation between floral design and pollen placement precision, even among taxa that have only recently been introduced, which indicates that flowers can achieve precise pollen placement on pollinators irrespective of previous association. Both of these conjectures could contribute to the higher (and less variable) precision of pollen placement by plant species visited by bats, compared with plant species visited by bees. More data are needed to test the validity of these hypotheses, particularly data from bat-pollinated plants, as one of the limitations of this study is the small sample size of bat-pollinated plants.

It is possible, however, for bee-pollinated species to achieve high precision as well. For example, we calculated pollen placement precision by three *Pedicularis* species on bumble bees based on the data reported in Armbruster *et al.* (2014), and values ranged from 0.469 to 0.734, which is considerably higher than

the average precision of 0.182 found in this study for bee-visited plants. Three factors that are likely to help *Pedicularis* achieve relatively precise pollen placement are (1) the style and anthers are contained within a galeate upper lip (Armbruster *et al.*, 2014), (2) they have a close evolutionary history with bumble bee pollinators (Macior & Ya, 1997; Huang & Fenster, 2007), and (3) bumble bees are relatively large bees. If our hypothesis about pollinator size relative to flower size is true, we would expect flowers to be able to achieve more precise pollen placement on larger bees such as bumble bees, compared with smaller bees such as honey bees. Other studies have also demonstrated that pollen deposition on the pollinator is influenced not only by floral design, but by pollinator size and fit with the flower (Moré *et al.*, 2006; Reyes *et al.*, 2016; Poblete Palacios *et al.*, 2019; Morais *et al.*, 2020).

Effect of floral symmetry on pollen placement

Our results demonstrate that zygomorphic flowers place pollen more precisely on bat and bee pollinators than actinomorphic flowers (Fig. S2). This result corroborates the finding of O'Meara *et al.* (2016) that symmetry was the most consistently influential factor associated with increased diversification rates among angiosperms; the authors inferred that zygomorphic flowers could have more opportunities for speciation due to precise pollen placement on pollinators, thus facilitating prezygotic reproductive isolation. It is unsurprising that symmetry plays such an important role in pollen placement, given that floral symmetry influences how pollinators approach flowers (Culbert & Forrest, 2016) and a more consistent approach can allow for more precise pollen placement (Fenster *et al.*, 2009). Our results are consistent with earlier studies demonstrating that zygomorphic flowers can achieve precise pollen placement on pollinators (Muchhala & Potts, 2007; Muchhala, 2007; Armbruster *et al.*, 2014; Huang *et al.*, 2015; Maguiña & Amanzo, 2016; Ruchisansakun *et al.*, 2016). Previous work has also shown that zygomorphic flowers exhibit less variance in flower size (Wolfe & Krstolic, 1999; Ushimaru *et al.*, 2007; Herrera *et al.*, 2008; Gong & Huang, 2009; Lázaro & Totland, 2014; Nikkeshi *et al.*, 2015) which can also contribute to precise pollen placement on the pollinator. While floral symmetry has been extensively studied previously, our findings are novel for comparing pollen placement by zygomorphic and actinomorphic flowers in the context of other floral traits predicted to influence pollen placement precision; we demonstrate that symmetry, in concert with these other floral traits, influences pollen placement precision on both large (nectar bat) and small (honey bee) pollinators.

Effect of corolla fusion on pollen placement

This study also demonstrates that plant species with fused corollas achieved greater precision when placing pollen on pollinators than plant species with unfused corollas (Fig. S2). The importance of sympetaly in plant–pollinator interactions has long been inferred (Darwin, 1862; Herrera *et al.*, 2008) and numerous studies have demonstrated the importance of morphological fit between corolla tube and pollinator (Nilsson, 1988; Muchhala, 2007; Muchhala & Thomson, 2009), but comparisons of plants with fused and unfused corollas are scarce (but please refer to Herrera *et al.*, 2008; Nikkeshi *et al.*, 2015). Stebbins (1951) noted that corolla fusion is most likely to be beneficial when paired with reduced stamen number and we did find a strong correlation between these two traits (Fig. S1). Additionally, Endress (1997) described corolla fusion as a key floral innovation and also noted that it often occurs concurrently with reduced stamen number, but did not discuss potential reasons why corolla fusion acts as a key innovation. Our results suggest that fused corollas constrain pollinator behaviour so that pollen is deposited more precisely, which can increase plant fitness by minimising pollen loss.

Effect of floral orientation on pollen placement

As predicted, flowers that were oriented horizontally or semipendant placed pollen more precisely than flowers that were oriented vertically (Fig. S2). We hypothesised that orientation would influence the precision of pollen placement as flowers that are oriented horizontally or semipendant constrain how pollinators approach flowers, unlike vertically oriented flowers that can be approached from any angle (Fenster *et al.*, 2009). Moreover, Armbruster & Muchhala (2020) demonstrated that bilaterally symmetric flowers are generally able to reorient after they have been disturbed from their normal orientation, while radially symmetric flowers are not. Such findings indicate that misorientation imposes a fitness cost to zygomorphic flowers, such as improper pollen placement on the pollinator, that is not experienced by actinomorphic flowers (Armbruster & Muchhala, 2020). Notably, we also observed that floral symmetry and orientation were highly correlated among our plant study species (Fig. S1). For example, in our bee data set, plant species with zygomorphic flowers were almost always oriented horizontally (11 species oriented horizontally and one species oriented vertically), while plants with actinomorphic flowers showed the reverse trend (16 species oriented vertically and four species oriented horizontally) ($\chi^2 = 15.468$, df = 1, $P < 0.001$ with Yates correction). Our results are therefore consistent with the finding of Armbruster & Muchhala (2020) that orientation is particularly important for zygomorphic flowers.

Effect of stamen : perianth (S : P) ratio on pollen placement

We provide an empirical demonstration that flowers with few stamens placed pollen more precisely than those with numerous stamens (Fig. S2), as has been generally assumed in the literature (Stebbins, 1951; Walker-Larsen & Harder, 2000; Sargent, 2004; O'Meara *et al.*, 2016). Indeed, O'Meara *et al.* (2016) found that reduced stamen number was one of three character states strongly associated with increased diversification rates and conjectured that reduced stamen number is favoured because it increases the precision of pollen placement, which our study confirmed. However, as previous authors have suggested, reduced stamen number is likely to be more effective when occurring in combination with other floral character states, such as sympetaly and zygomorphy (Stebbins, 1951; Endress, 1997). The strong correlations observed in this study between S : P ratio and the other three floral traits

(Fig. S1) make sense in terms of the pollination-precision hypothesis; flowers that direct pollinator behaviour through bilateral symmetry, a fused corolla, or horizontal floral orientation benefit from increasing pollen amounts in the few stamens that consistently contact pollinators and elimination of the other stamens that do not contribute to successful pollination, which can lead to the evolution of reduced stamen number.

Conclusions

The pollination-precision hypothesis predicts that flowers with bilateral symmetry, fused corollas, horizontal or semipendant orientation and few stamens should place pollen more precisely on the pollinator's body compared with flowers with alternative floral character states. Our study provides empirical support for this hypothesis using phylogenetically corrected analyses with data from two very different pollinator groups, honey bees (genus *Apis*) and nectar bats (family Pteropodidae). Moreover, we found that pollen placement is more precise and less variable overall on bat pollinators than bee pollinators. We hypothesise that the difference between bats and bees is due to their different sizes, as precise pollen placement should be easier to achieve on large pollinators than small pollinators, relative to flower size. Our findings help to explain the prevalence of specific floral character states that enhance precise pollen placement on the pollinator. These character states potentially increase plant fitness through greater efficiency of pollen transfer and plant diversification through reduced interspecific pollen transfer.

Author contributions

All authors contributed to research design; ABS collected data; ABS analysed the data with input from CD, CBF and MRD; ABS wrote the first draft and all authors helped revise the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Correlations between floral traits for plant study species.

Fig. S2 Correlations between pollen placement precision and floral traits for plant study species.

Notes S1 Results of analyses in which pseudanthial plant species were excluded from data sets.

Table S1 Details about each plant species visited by nectar bats.

Table S2 Details about each plant species visited by honey bees.

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