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Remarkable variation in androecial morphology is closely associated with corolla traits in Western Hemisphere Justiciinae (Acanthaceae: Justicieae)

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- **Background and Aims** Few studies of angiosperms have focused on androecial evolution in conjunction with evolutionary shifts in corolla morphology and pollinator relationships. The Western Hemisphere clade of Justiciinae (Acanthaceae) presents the rare opportunity to examine remarkable diversity in staminal morphology. We took a phylogenetically informed approach to examine staminal diversity in this hypervariable group and asked whether differences in anther thecae separation is associated with phylogenetically informed patterns of variation in corolla morphology. We further discuss evidence for associations between anther diversity and pollinators in this lineage.
- **Methods** For the *Dianthera*/*Sarotheca*/*Plagiacanthus* (DSP) clade of Western Hemisphere Justiciinae, we characterized floral diversity based on a series of corolla measurements and using a model-based clustering approach. We then tested for correlations between anther thecae separation and corolla traits, and for shifts in trait evolution, including evidence for convergence.
- **Key Results** There is evolutionary vagility in corolla and anther traits across the DSP clade with little signal of phylogenetic constraint. Floral morphology clusters into four distinct groups that are, in turn, strongly associated with anther thecae separation, a novel result in Acanthaceae and, to our knowledge, across flowering plants. These cluster groups are marked by floral traits that strongly point to associations with pollinating animals. Specifically, species that are known or likely to be hummingbird pollinated have stamens with parallel thecae, whereas those that are likely bee or fly pollinated have stamens with offset, divergent thecae.
- **Conclusions** Our results suggest that anther thecae separation is likely under selection in concert with other corolla characters. Significant morphological shifts detected by our analyses corresponded to putative shifts from insect to hummingbird pollination. Results from this study support the hypothesis that floral structures function in an integrated manner and are likely subject to selection as a suite. Further, these changes can be hypothesized to represent adaptive evolution.

Key words: Acanthaceae, anther diversity, covariation, floral morphology, hummingbird pollination, insect pollination, *Justicia*, phylogenetic comparative methods.

INTRODUCTION

Across flowering plants, the macromorphological traits of flowers and their roles in pollination biology have received a great deal of study (Faegri and van der Pijl, 1979; Temeles and Kress, 2003; Fenster *et al.*, 2004; Temeles *et al.*, 2013; Lagomarsino *et al.*, 2017; Smith and Kriebel, 2018). For example, multiple studies have associated differences in corolla colour (Grant and Grant, 1968; Melendez Ackermann *et al.*, 1997; Wilson *et al.*, 2004) and corolla tube length with differences in pollinators (Grant and Temeles, 1992; Whittall and Hodges, 2007; Muchhala and Thomson, 2009). Interestingly, although pollen-producing stamens and pollen-receiving stigmas are entirely critical for successful reproduction, these structures have received far less study (but see Bernhardt, 1996; Armbruster *et al.*, 2009; Rosas-Guerrero *et al.*, 2011; Kriebel *et al.*, 2019, 2020).

Stamens are the pollen-producing organs of flowers, collectively termed the androecium, and can vary considerably both within and among flowers. Stamen morphology, number and function have been modified throughout angiosperm evolution. Stamens are especially morphologically diverse among early-diverging flowering plants (Endress, 2011). In these lineages, floral organs are not always fixed either morphologically or in number, and transitions between floral organs can be gradual (e.g. waterlilies, Nymphaeaceae; Endress, 2011). In other early-diverging angiosperms (e.g. Annonaceae), there is differentiation in function across the androecium: innermost stamens may be sterile, may provide food resources for pollinating insects, or may secrete exudates that adhere pollen to insect bodies (Endress, 1984; Li and Xu, 2019; Saunders, 2020). In contrast to the situation in many early-diverging angiosperms, in most monocots and eudicots, and regardless of whether flowers are actinomorphic or zygomorphic, stamens are relatively few, well differentiated from the other floral organs, and identical within a flower. In these groups, stamens are generally rather stereotypical in form, with a sterile filament that apically bears two

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thecae that are more or less parallel to each other. Each theca typically has two pollen sacs and opens by a longitudinal slit between the two pollen sacs (Endress, 2011).

Exceptions to the dominant pattern of intrafloral homogeneity of stamens include heteranthery (i.e. having two or more morphologically distinct stamen types that may also differ in function in the same flower), a trait that has evolved multiple times in at least 20 families across 12 orders (Vallejo-Marín *et al.*, 2010), often in association with bee pollination (Vogel, 1978; Endress, 1994; Jesson and Barrett, 2003). In heterantherous species, stamens differ in position, colour, shape and mode or timing of dehiscence (Dellinger *et al.*, 2019; Kay *et al.*, 2020; Barrett, 2021). In most cases, one or more of the stamens functions in pollination and the others provide food or some other attraction for flower-visiting animals. This 'division of labour' concept seems to fit many cases of heteranthery but not all. For example, it has also been suggested that heteranthery can serve as a mechanism to release pollen conservatively, thus promoting pollen export and siring success (Kay *et al.*, 2020).

The vast majority of Asteridae have flowers with a single whorl of stamens with little variation among them other than size. Among the exceptions are heterantherous Solanaceae and Ericaceae, the latter with inverted anthers and poricidal dehiscence (Barrett, 2021). Another notable exception to intrafloral staminal homogeneity is the case of some Lamiaceae in which the thecae are highly divergent and separated by elaborated connective tissue. Thus modified, the paired stamens operate like levers to interact with the bodies of pollinators. This phenomenon has been relatively well studied, with phylogenetic analyses indicating that, in *Salvia* and relatives, this elaborate pollination mechanism may be lost as well as gained (Walker and Sytsma, 2007). Further, these transitions are correlated with pollinator shifts (Walker *et al.*, 2004; Walker and Sytsma, 2007; Kriebel *et al.*, 2019, 2020). For example, in *Hemigenia*, melittophilous species generally have stamens with lever mechanisms, whereas ornithophilous species typically have immobile stamens (Guerin, 2005; Westerkamp and Claßen-Bockhoff, 2007).

The relative paucity of diversity among stamens holds true across much of the large (~4000–5000 species) plant family Acanthaceae (Lamiales, Asteridae) (Manzitto-Tripp *et al.*, 2022). In Acanthaceae, stamens vary across major lineages in number (two to four), but still are almost always typical stamens with parallel thecae. In contrast, among species of the tribe Justiciinae, there is remarkable androecial diversity that rivals that found in Lamiaceae. This diversity has never been studied from either a functional (i.e. pollination biology) or evolutionary perspective.

Justiciinae consists of the hyperdiverse genus *Justicia* (~700 species) plus at least 15 other genera from the Eastern and Western Hemispheres (EH and WH, respectively) (Kiel *et al.*, 2017; Manzitto-Tripp *et al.*, 2022). Members of this lineage have an androecium composed of two bithecous stamens that are mirror imaged but otherwise identical. With few exceptions, these plants exhibit some form of what we have called anther 'complexity' in earlier papers: anthers have thecae that may be unequal in size and shape (Fig. 1C), inserted at different levels on the filament's connective, oriented along the filament in different directions (Fig. 1B–D, H, J), and/or appendaged (Fig. 1B, D, H, J) (McDade *et al.*, 2000; Kiel *et al.*, 2017, 2018). Androecial diversity is especially apparent in the species-rich (~400 species) WH Justiciinae lineage (Fig.

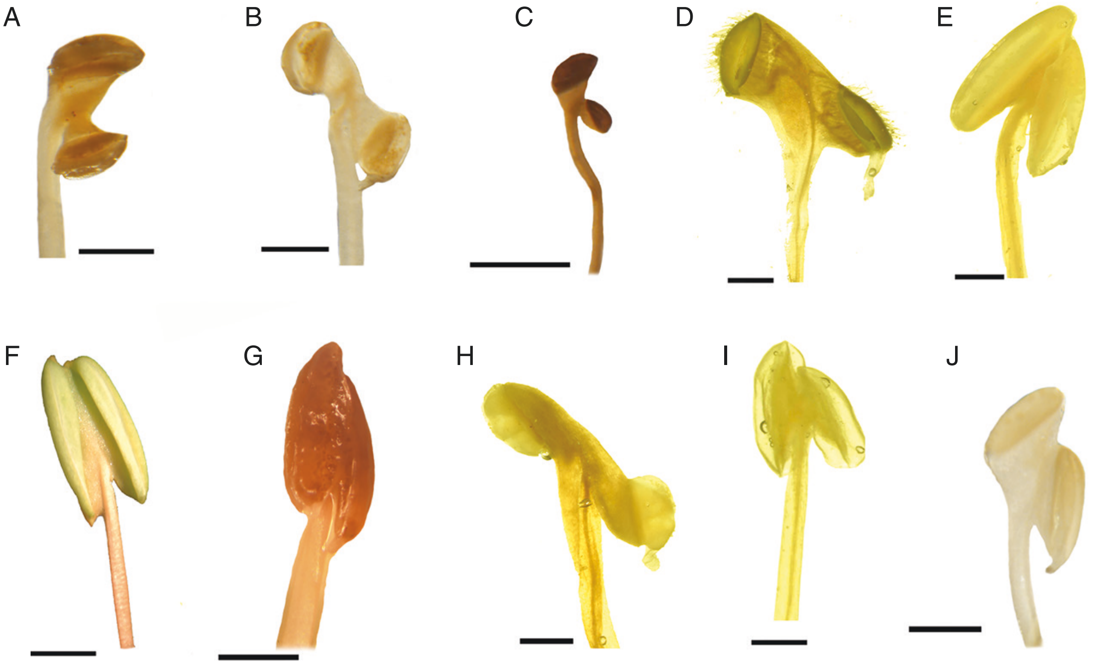

Fig. 1. Examples of anther diversity among species of *Justicia* in the DSP clade. (A) *J. filibracteolata* (*Kiel and Saab 265,* Colombia), (B) *J. trichotoma* (*Kiel 277,* Costa Rica), (C) *J. comata* (*Kiel and Saab 250,* Colombia), (D) *J. salviiflora* (*Kiel et al. 235,* Mexico), (E) *J. torresii* (*Torres and Cortez 11472,* Mexico), (F) *J. spicigera* (*Kiel s.n.* cultivated), (G) *J. adenothyrsa* (*Kiel et al. 68*, Mexico), (H) *J. kuntzei* (*Lozano et al. 1481,* Bolivia), (I) *J. sonorae* (*Van Devender 98-434*, Mexico), (J) *J. ovata* (*Kiel and Fisher 246*, United States). Scale bars: (A–C, J) = 0.5 mm; (D–I) = 1.0 mm.

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1). In all phylogenetic work to date, this lineage is strongly supported as monophyletic and comprises remarkable floral morphological diversity (Fig. 2; see also Fig. 1 in Kiel *et al.*, 2018). Flowers range from a few mm to 14 cm in length and in colour from white to various shades of purple, yellow, orange, pink and red. Bees, lepidopterans, bats and hummingbirds have been documented as pollinators or visitors to flowers of WH Justiciinae (Michener, 1979; McDade and Kinsman, 1980; McMullen,1994; Daniel, 2004; McDade and Weeks, 2004; Vogel *et al.*, 2004; Döll *et al.*, 2007; Quintana-Vásquez, 2007; Schmidt-Lebuhn *et al.*, 2019; C. A. Kiel, pers. obs.; A. E. Fisher, pers. obs.).

Kiel *et al.* (2018) showed that floral traits exhibit remarkable evolutionary vagility across WH Justiciinae. Notably, there have been at least 14 transitions to traits consistent with hummingbird pollination from a bee-/fly-pollinated ancestor. Based on our observations, plants in the WH Justiciinae lineage that have been documented or inferred to be hummingbird pollinated tend to have anthers with thecae that are parallel or nearly so (Fig. 1E–G, I; Kiel *et al.*, 2018). In contrast, flowers of species that are documented or inferred to be insect pollinated tend to have anthers with expanded connective tissue that results in varying degrees of offset or super-positioned thecae, with one or both thecae often also bearing spur-like structures basally (Fig. 1A–D, H, J). The association between anther traits and other floral features yields the hypothesis that relationships with pollinating animals have contributed to the evolution of anther morphological diversity in this lineage.

Here, we take a phylogenetically informed approach to examine the remarkable diversity in staminal morphology in the WH clade of Justiciinae (Acanthaceae). We also place this androecial diversity into the context of interspecific variation in corolla morphology. We test the hypothesis that anther and corolla morphological traits covary in *Justicia.* We apply morphometric methods, a clustering approach, and phylogenetically corrected statistical methods to characterize corollas. WH Justiciinae comprise nine major clades based on the most recent study of 134 taxa (Kiel *et al.*, 2018). We focus here on the morphologically diverse *Dianthera/Sarotheca/Plagiacanthus* (DSP) clade (*sensu* Kiel *et al.*, 2018), a lineage of at least 29 species that together encompass a wide range of corolla shapes and sizes (Fig. 2) and anther morphologies (Fig. 1) to address the following questions. (1) How have anther traits evolved with respect to phylogeny in the DSP clade? (2) Is anther

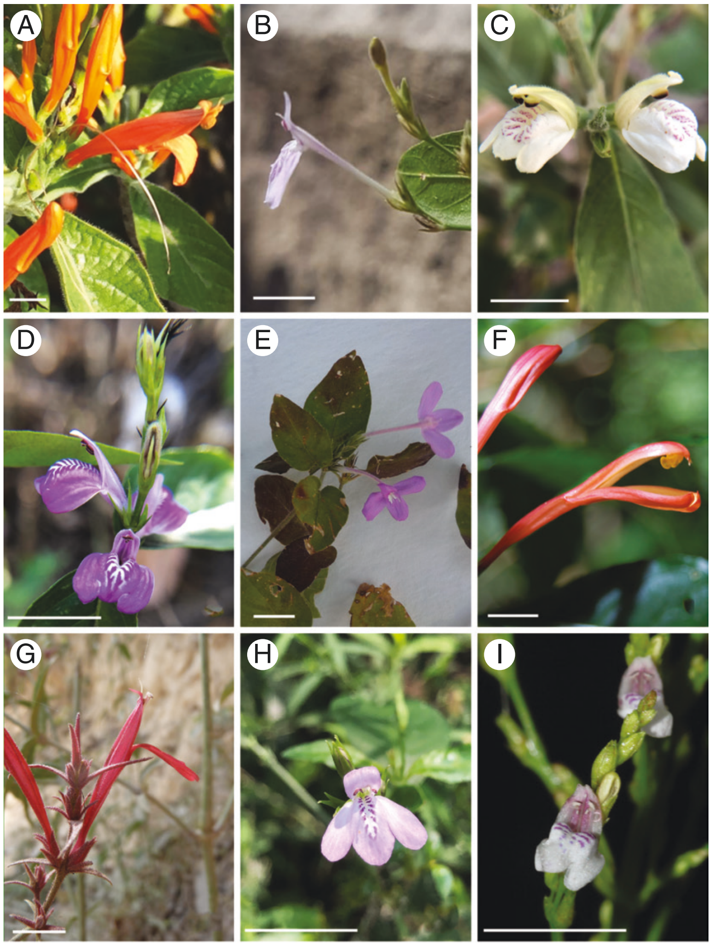

Fig. 2. Examples of corolla diversity in the DSP clade. (A) *Justicia spicigera* (*Kiel s.n.*, cultivated), (B) *J. ramosa* (*Tripp and Medina 9675*, Mexico), (C) *J. salviiflora Kiel et al. 235*, Mexico, (D) *J. breviflora* (*Kiel et al. 239*, Mexico), (E) *J. hilsenbeckii* (*Kiel et al. 56,* Mexico), (F) *J. macrantha* (*Kiel and McDade 79*, Costa Rica*),* G. *J. adenothyrsa* (*Kiel et al.* 68, Mexico), H. *J. ovata,* (*Kiel and Fisher 246,* United States), I. *J. comata* (*Kiel and Saab 250*, Colombia). All photos by C. Kiel except (B) by E. Tripp. Scale bars: (A–G) = 1.0 cm; (H, I) = 0.4 cm.

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thecae separation correlated with other floral traits? (3) Can the patterns of trait variation across this lineage be explained by phylogenetic history? (4) If correlations between corolla traits and anther morphology exist, is there evidence for patterns of convergence of these traits? (5) Is variation in floral form consistent with evidence for pollinator relationships suggesting that patterns of trait variation may be explained by selection by pollinators? This is the first study of Acanthaceae to examine variation in androecial traits in a comparative phylogenetic context and to relate that variation to corolla form. Results from this research provide a foundation for broad-scale study across the entire WH Justiciinae and for ecological studies of the role of the morphological variation documented here in pollination.

MATERIALS AND METHODS

Sampling

To test the hypothesis that there is covariation between anther and corolla morphological traits, we selected 29 taxa from the DSP clade (Kiel *et al.*, 2018). Among species, corolla colour ranges from white-green to lilac to purple or pink to orange to red (Fig. 2), and corolla size ranges from 0.5 to 4.5 cm long. Collectively, the lineage is widespread, with species occurring in diverse habitats from temperate wet areas in the USA to tropical dry forests in Mexico and to tropical wet forests in Central and South America. Phylogenetic relationships across this clade have been studied using DNA sequence data and there is relatively strong support from both maximum likelihood bootstrap and Bayesian posterior probability for most aspects of relationship (Kiel *et al.*, 2018)

Three accessions were sampled for each of the 29 taxa; *Justicia concavibracteata* was the exception as only two accessions suitable for sampling were available of this Peruvian endemic (Supplementary Data S1). Although larger sample sizes would have been desirable, for most species in the DSP clade suitable floral material was limited. Many species of WH *Justicia* have narrow distributions, are uncommon or sparsely distributed such that specimens are few (exceptions include *J. americana*, *J. comata* and *J. pectoralis*). Evidence suggests that, in most cases, a sample size of three corollas is adequate: there is little intraspecific variation among anthetic corollas in most species of *Justicia* or other Acanthaceae with which we have worked. For the vast majority of *Justicia*, thoroughly documented floristic and taxonomic works report narrow ranges of values for morphometric traits of flowers (but not for leaves and other structures) (Daniel, 1995, 2004; Wasshausen and Wood, 2004; Wasshausen, 2006; Darbyshire *et al.*, 2010; Kiel, 2020). Further we note that other studies at the macroevolutionary level have addressed similar questions using fewer than three vouchers per species (e.g. Lagomarsino *et al.*, 2017; Smith and Kriebel, 2018) or using median values from published taxonomic treatments or even measurements from illustrations (e.g. Kriebel *et al.*, 2020, 2022).

Of necessity, we used floral material from several sources including field-collected tissues stored in FPA (formalin propionic alcohol), one greenhouse accession (*J. spicigera*, also stored in FPA), and herbarium specimens. For a subsample of four species, we tested whether preservation method (i.e. FPA versus drying, same collection) resulted in significant differences in floral measurements. We found no significant differences between floral measurements regardless of how flowers were preserved (Supplementary Data S2). We thus feel confident in ascribing differences that we detect to interspecific variation rather than to preservation artefacts.

Measurements

Corollas and anthers were imaged at California Botanic Garden with a camera-mounted Leica M165 dissecting microscope (Leica Microsystems Inc., Bannockburn, IL, USA), using Olympus cellSens Standard 1.15 (©2009–2016) software. Figure 3 illustrates how the following corolla traits were measured: (1) corolla length (straight-line distance from the base of the tube where it inserts into the base of the calyx to the tip of the upper lip); (2) corolla tube length (straight-line distance from the base of the tube to where the lower lip diverges from the tube); (3) corolla tube width at midpoint of the tube; (4) width of corolla mouth opening; (5) upper lip length; (6) lower lip width; (7) lower lip length. To capture the degree of separation, if any, between the two thecae of the same stamen, two additional measurements were taken: from the apex of the distal theca to the apex of the proximal theca (shown as the distance between points A and B in Fig. 3), and from the apex of the outer theca to the base of the inner theca (shown as the distance between points A and C in Fig. 3). We devised a metric, the ratio (A–B):(A–C), to capture the degree of separation between thecae. Ratio values close to 0 correspond to thecae that are nearly parallel, whereas values close to 1 indicate markedly separated thecae. Anther appendages (see examples shown in Fig. 1B, D, G–H, J), if present, were not included in the measurements because these structures are interpreted as extensions of the connective tissue (i.e. tissue between the thecae that connects the filament to the anthers) rather than part of the thecae (C. A. Kiel, pers. obs.). All measurements were acquired digitally to the nearest 0.1 mm using ImageJ version 1.43r (Schneider *et al.*, 2012). We also crosschecked our measurements with comprehensive taxonomic treatments that include species from the DSP clade sampled here (Hilsenbeck, 1990; Daniel, 1995, 2004; Wasshausen and Wood, 2003). In all cases, data collected here are consistent with those reported in the literature. Linear metrics were normalized through log transformation, whereas ratios were arc-sin transformed.

Phylogenetic and divergence time analyses

The seven-gene alignment generated for Kiel *et al.*'s (2018) comprehensive study of WH Justiciinae was used to generate a phylogenetic framework (available in Dryad: https://doi.org/10.5061/dryad.5377b8k). The seven loci were *trnS-G*, *trnT-L*, *ndhF-trnL*$_{\text{(UAG)}}$, *ndhA*, *rpl16*, nuclear ribosomal internal transcribed spacer region (ITS1, ITS2 and 5.8S), and the low-copy nuclear marker *PhotI*. To construct ultrametric trees for downstream analyses, we estimated divergence times in BEAST2 version 2.2.5 (Bouckaert *et al.*, 2014) using the full dataset from Kiel *et al.* (2018). Details of settings and fossil calibrations for the BEAST analysis are as in Supplementary Data

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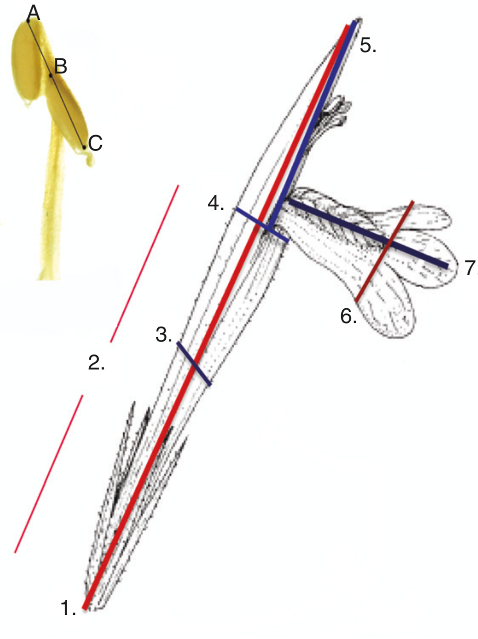

Fig 3. Eight traits measured and analysed for this study. 1, Corolla length; 2, corolla tube length; 3, corolla tube width at midpoint of the tube; 4, width of mouth opening; 5, upper lip length; 6, lower lip width; 7, lower lip length; 8, anther thecae separation measurement [ratio between the distances (A–B) and (A–C), calculated as (A–B)/(A–C)].

Fig. S1. The BEAST runs were merged using LogCombiner 2.5.2 (Bouckaert *et al*., 2014), and a maximum clade credibility (MCC) tree with median ages and 95 % highest posterior density was generated with TreeAnnotator 2.5.2 (Bouckaert *et al*., 2014). Along with the MCC tree, we arbitrarily sampled 100 trees from the posterior distribution to examine phylogenetic uncertainty in downstream analyses. All taxa, with the exception of the 29 focal taxa from the DSP clade, were pruned from the MCC tree and from the 100 trees sampled from the posterior distribution in R.

Comparative analyses

All comparative analyses were conducted in R version 3.6.0 (R Core Team, 2020) using RStudio version 1.1.463 (2019). Correlation plots were constructed to identify patterns of covariation among the seven corolla traits with the goal of identifying those that vary independently. Using the Pearson correlation coefficient, we considered pairs of variables with $r > 0.85$ to be strongly correlated. Based on this analysis, five corolla traits were retained for further analyses (see below).

Characterization of corollas

To initially group floral form based on the five corolla measurements retained (anther traits not included), we performed a model-based clustering approach using maximum likelihood estimation and the Bayesian information criterion (BIC) to identify the most likely model and number of clusters using the *mclust* function in the package mclust (Fraley *et al*., 2016). The optimal model was selected according to BIC initialized by hierarchical clustering for parameterized Gaussian mixture models. Principal component analysis (PCA) was then used to characterize variation in thecae separation and corolla traits among species in multidimensional space.

Model fitting of traits and trait correlations

Mean transformed values for the five corolla traits and for anther thecae separation ratio were calculated for each of the 29 species, reducing the total number of measurements from 86 samples to means for each of 29 taxa. Initial examination of cluster groupings of floral form in relation to thecae ratios were conducted using box and whisker plots to determine whether characters were sufficiently variable, and to identify outlying data points. A one-way ANOVA and Tukey's honestly significant difference (HSD) test and a phylogenetic ANOVA were conducted, with cluster group as a categorical variable, to test whether variation in anther thecae separation among cluster groups exceeded variation expected by chance. The phylogenetic ANOVA consisted of 1000 simulations coupled with a Holm *post hoc* test in phytools (Revell, 2012).

We first conducted model fitting for anther thecae separation using Geiger (Harmon *et al*., 2008). The evolutionary models examined were (1) Brownian motion (BM): trait evolution under a random walk process with a constant rate (Felsenstein, 1985); and (2) an Ornstein–Uhlenbeck (OU) model that includes a selection parameter that pulls trait evolution towards one or more optimal values (Martins, 1994). Since the corrected Akaike information criterion (AICc) is recommended in studies with sample sizes < 40 (Symonds and Moussalli, 2011), this criterion, along with likelihood (Burnham and Anderson, 2002), was used to select the best-fit model of trait evolution.

To estimate correlations between thecae separation ratios (response variable) and individual corolla traits while accounting for phylogenetic relatedness, we used the phylogenetic generalized least squares (PGLS) method (Martins and Hansen, 1997) in R package nlme (Pinheiro *et al*., 2019). These analyses incorporated the MCC tree and were repeated across the posterior sample of 100 trees.

Phylogenetic signal

To examine whether patterns of trait variation may be attributed to phylogenetic history, we quantified phylogenetic signal by estimating Blomberg's *K* (Blomberg *et al*., 2003) for corolla traits and for thecae separation. Blomberg's *K* was quantified using phytools (Revell, 2012), and then tested for significance from zero by comparing it with *K* values for 10 000 null models that randomly shuffle taxa across the tips of the phylogeny using the *phylosig* function. This analysis was repeated across the 100 trees sampled from the posterior distribution to account for phylogenetic uncertainty.

Character mapping/ancestral state reconstructions

The *contmap* function of the phytools package (Revell, 2012) was implemented to visualize changes in anther thecae

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separation in comparison with correlated floral traits from the PGLS analyses. The corolla traits correlated with anther thecae separation, in conjunction with their clustering groups, were then further explored in phylomorphospace using phylogenetic PCA (pPCA) in phytools (Revell, 2009, 2012).

Evolutionary regime shifts

We examined models that tested the hypothesis that anther thecae separation and corolla traits evolve towards different optima that correspond to the four cluster groups identified by the mclust analyses. We fitted four models of trait evolution using mvMORPH (Clavel *et al*., 2015). These included univariate (single trait) and multivariate models (corolla traits correlated with anther thecae separation, examined as a suite) with constant rates: a single-rate BM process and a single-optimum OU (OU1) model. We also implemented a Brownian Motion model (BMM) model that allows for each regime to assume a different drift rate, σ^2, and a multiple-optimum OU (OUM) model that permits different optima with single values for α, selective strength, and σ^2, rate of stochastic motion, across all selective regimes. The OUM model examined included the specified mclust clustering groups as an *a priori* selective regime. The evolutionary histories of these *a priori* regimes were estimated on the tree prior to model fitting using stochastic character mapping with the *make.simmap* function in phytools (Revell, 2012). Model fit was evaluated using AICc and AICc weights (Burnham and Anderson, 2002; Burnham *et al*., 2011). These analyses were repeated across the 100 trees sampled from the posterior distribution to account for phylogenetic uncertainty.

Shifts in trait evolution and convergence

To investigate selection on multivariate floral traits in the DSP clade, we examined modelled shifts in correlated floral traits under an OU process. We applied the package `l1ou` (Khabbazian *et al*., 2016) to correlated multivariate floral traits to estimate potential convergence onto phenotypic optima without *a priori* designation of selective regimes. We used the phylogenetic Bayesian information criterion (pBIC), a modification of BIC that allows for phylogenetic correlation of shifts, when selecting the best-fit shift configuration model. Analyses included the MCC tree and the posterior distribution of 100 trees. For comparison, and to further explore our data and other models, we re-ran the analyses using the AICc. We performed these methods on the raw traits with a maximum of 20 shifts, and calculated support for each shift by running 1000 bootstrap iterations.

RESULTS

Phylogenetic results from the BEAST analysis were similar to results of Kiel *et al*. (2018), which used maximum likelihood and Bayesian criteria (Supplementary Data Fig. S1). All of the ten major clades of the WH Justiciinae lineage from Kiel *et al*. (2018) were recovered and relationships among species in the DSP clade were well resolved with most nodes strongly supported (i.e. ≥ 0.95 Bayesian posterior probability) (Supplementary Data Fig. S2; see also Kiel *et al*., 2018).

Measurements for all traits and voucher information for individual taxa are provided in Supplementary Data S1; mean values are summarized in Table 1. Among corolla traits measured, two pairs were strongly correlated: (1) corolla length and corolla tube length ($r = 0.94$); and (2) upper lip length and lower lip length ($r = 0.90$). Corolla length and upper lip length were thus removed from subsequent analyses.

The mclust clustering analysis of the five retained corolla traits estimated that four clusters optimally accommodate the variation in floral form. The best-fit model for 86 samples based on BIC (BIC = 247.67) was the VEV model (ellipsoidal, equal shape). Table 2 summarizes the four clusters by taxon and raw trait means.

Results of the PCA of five corolla traits plus anther thecae separation showed that the first three PCs accounted for 54.3, 19.6 and 15.1 % of the total variance, respectively (Fig. 4; Supplementary Data S3). Positive loadings on PC1 were contributed by anther thecae separation, whereas all corolla traits had negative loadings. PC2 reflected high positive loadings for anther thecae separation and traits associated with corolla width (i.e. corolla mouth width, midpoint of corolla tube width). PC3 had the highest loadings for lower lip width.

A box and whisker plot summarizing anther thecae separation among clusters is shown in Fig. 5. The phylogenetic ANOVA returned a significant relationship between anther thecae separation and cluster based on floral morphometric traits (Table 3, $P < 0.001$). Holm's *post hoc* test revealed that species of cluster 1 were significantly different in anther thecae separation from those of cluster groups 2 and 3, but not 4 ($P = 0.52$). Species of cluster 2 differed significantly in anther separation from clusters 1 and 4 but not 3. Overall, similar results were found from the standard one-way ANOVA and Tukey's HSD *post hoc* test (Supplementary Data S4).

Across anthers and corolla traits, Blomberg's K statistic ranged from 0.44 to 0.83, indicating variation in degree of phylogenetic signal (Table 4). Most traits were statistically indistinguishable from $K = 0$ ($P > 0.05$), consistent with little phylogenetic signal (i.e. thecae separation, midpoint of corolla tube width, corolla mouth width, lower lip width and lower lip length). Blomberg's K for corolla tube length ($K = 0.83$) was significantly different from zero. These results were consistent across the posterior distribution of 100 trees (Table 5).

For the PGLS analyses, anther thecae separation was best fitted by the OU model in the MCC tree and in 65 of the 100 trees in the posterior distribution. The PGLS analyses under the OU model (Supplementary Data Fig. S3; Table 5) indicated a strong relationship between anther thecae separation and corolla tube length as well as lower lip length. These significant relationships were recovered in all PGLS analyses replicated across the sample of 100 trees from the posterior distribution (Table 5). In contrast, corolla traits not significantly correlated with thecae separation under the OU model included corolla mouth opening width, lower lip width and corolla tube width.

Ancestral state reconstruction (Fig. 6) suggested marked shifts in anther thecae separation, corolla tube length and lower lip length across the DSP clade. Anther thecae separation has shifted at least four times, from the predicted ancestral condition of anther thecae separated to anther thecae

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Table 1. *Variation in eight morphological characters for 29 species in the DSP clade: mean, standard error.* N = *3 for all species except* J. concavibracteata *(*N = *2). CL, corolla length (mm); TL, corolla tube length (mm); ULL, upper lip length (mm); LLW, lower lip width (mm); LLL, lower lip length (mm); TDR, thecae separation ratio.*

Species	CL	TL	Midpoint	Mouth	ULL	LLW	LLL	TDR
J. adenothyrsa	34.4 ± 0.95	22.4 ± 0.78	2.67 ± 0.17	3.67 ± 0.17	12.23 ± 0.50	5.27 ± 0.12	13.53 ± 0.90	0.30 ± 0.02
J. alpina	20.3 ± 0.17	10.43 ± 0.30	2.77 ± 0.15	4.77 ± 0.15	9.5 ± 0.30	11.66 ± 0.88	14.5 ± 0.29	0.52 ± 0.01
J. americana	11.13 ± 0.59	3.0 ± 0.0	3.17 ± 0.01	3.77 ± 0.12	6.93 ± 0.07	6.70 ± 0.15	7.53 ± 0.12	0.43 ± 0.02
J. boliviana	11.5 ± 1.32	4.7 ± 0.15	1.7 ± 0.06	2.3 ± 0.06	5.13 ± 0.07	7.06 ± 0.19	5.36 ± 0.22	0.55 ± 0.06
J. breviflora	11.9 ± 0.32	4.57 ± 0.23	2.13 ± 0.33	3.57 ± 0.03	6.37 ± 0.38	8.20 ± 0.64	3.50 ± 0.29	0.48 ± 0.03
J. calycina	41.27 ± 1.18	22.3 ± 0.46	3.1 ± 0.06	6.73 ± 0.15	20.57 ± 0.38	4.20 ± 0.40	20.53 ± 0.24	0.19 ± 0.03
J. candelarae	9.37 ± 0.12	5.00 ± 0.58	2.60 ± 0.10	2.87 ± 0.09	3.93 ± 0.07	6.37 ± 0.08	5.9 ± 0.06	0.56 ± 0.01
J. colorifera	36.23 ± 1.0	21.7 ± 0.17	2.00 ± 0.0	5.35 ± 0.05	10.43 ± 0.20	4.50 ± 0.15	10.40 ± 0.12	0.29 ± 0.01
J. comata	5.83 ± 0.35	1.4 ± 0.06	0.93 ± 0.15	1.77 ± 0.15	2.9 ± 0.36	2.00 ± 0.29	3.50 ± 0.15	0.58 ± 0.02
J. concavibracteata	6.35 ± 0.05	3.55 ± 0.05	1.00 ± 0.0	2.55 ± 0.05	3.65 ± 0.15	4.00 ± 0.0	3.35 ± 0.12	0.53 ± 0.01
J. filibracteolata	23.5 ± 1.47	10.33 ± 0.33	2.93 ± 0.07	3.83 ± 0.17	11.87 ± 0.13	8.93 ± 0.12	12.93 ± 0.18	0.68 ± 0.03
J. galapagana	10.13 ± 0.13	5.50 ± 0.29	1.53 ± 0.03	3.13 ± 0.13	4.4 ± 0.10	5.83 ± 0.20	4.30 ± 0.12	0.40 ± 0.0
J. hilsenbeckii	23.16 ± 0.60	17.67 ± 0.33	0.92 ± 0.08	1.1 ± 0.06	4.9 ± 0.10	11.33 ± 0.18	6.77 ± 0.15	0.24 ± 0.01
J. kuntzei	18.63 ± 0.19	6.83 ± 0.17	3.03 ± 0.03	4.33 ± 0.17	10.57 ± 0.23	6.33 ± 0.09	9.27 ± 0.15	0.73 ± 0.06
J. lanceolata	13.13 ± 0.13	4.53 ± 0.12	1.27 ± 0.03	2.13 ± 0.09	4.3 ± 0.15	5.97 ± 0.09	7.37 ± 0.23	0.40 ± 0.05
J. macrantha	48.43 ± 0.61	25.3 ± 0.17	4.00 ± 0.12	4.67 ± 0.12	25.03 ± 0.12	10.2 ± 0.12	23.9 ± 0.29	0.21 ± 0.03
J. mendax	23.33 ± 0.73	18.33 ± 0.33	2.00 ± 0.0	4.03 ± 0.03	10.3 ± 0.11	6.77 ± 0.15	11.33 ± 0.18	0.29 ± 0.07
J. metallica	10.83 ± 0.44	8.30 ± 0.15	0.90 ± 0.06	1.43 ± 0.07	3.77 ± 0.15	3.47 ± 0.03	3.63 ± 0.19	0.46 ± 0.01
J. oerstedii	26.47 ± 0.38	15.17 ± 0.09	3.00 ± 0.06	6.53 ± 0.26	12.23 ± 0.07	6.13 ± 0.03	14.07 ± 0.12	0.34 ± 0.01
J. ovata	8.16 ± 0.07	4.64 ± 0.10	0.89 ± 0.11	1.87 ± 0.12	3.70 ± 0.12	3.4 ± 0.06	4.73 ± 0.15	0.40 ± 0.06
J. pectoralis	6.9 ± 0.42	3.57 ± 0.12	1.10 ± 0.06	1.63 ± 0.09	2.50 ± 0.06	2.5 ± 0.06	2.87 ± 0.07	0.55 ± 0.01
J. ramosa	24.33 ± 0.65	15.0 ± 0.61	0.83 ± 0.07	0.90 ± 0.08	5.27 ± 0.12	7.77 ± 0.15	5.00 ± 0.0	0.38 ± 0.02
J. salviiflora	16.43 ± 0.56	8.27 ± 0.45	4.00 ± 0.50	6.90 ± 0.15	7.27 ± 0.50	7.83 ± 0.79	7.36 ± 0.56	0.62 ± 0.01
J. sonorae	28.4 ± 0.55	22.8 ± 1.01	2.0 ± 0.12	5.07 ± 0.07	8.23 ± 0.33	12.43 ± 0.58	10.36 ± 0.23	0.23 ± 0.02
J. spicigera	43.7 ± 0.78	25.9 ± 0.80	5.03 ± 0.09	6.13 ± 0.38	17.47 ± 0.23	5.1 ± 0.05	22.26 ± 0.09	0.21 ± 0.02
J. tenusitachys	27.33 ± 0.73	13.6 ± 0.23	3.10 ± 0.10	4.00 ± 0.12	12.30 ± 0.17	3.23 ± 0.15	14.6 ± 0.31	0.29 ± 0.06
J. torresii	32.33 ± 1.45	21.47 ± 0.31	3.00 ± 0.0	5.13 ± 0.09	9.80 ± 0.25	4.33 ± 0.33	9.53 ± 0.30	0.20 ± 0.0
J. trichotoma	16.33 ± 0.10	10.10 ± 0.06	0.88 ± 0.11	3.30 ± 0.06	7.30 ± 0.12	7.00 ± 0.0	6.73 ± 0.12	0.74 ± 0.0
J. vernalis	11.26 ± 0.52	5.23 ± 0.54	2.90 ± 0.06	2.93 ± 0.07	3.50 ± 0.23	5.57 ± 0.18	4.40 ± 0.26	0.64 ± 0.02

that are parallel or nearly so (i.e. as observed in *J. calycina*, *J. colorifera*, *J. hilsenbeckii*, *J. macrantha*, *J. sonorae*, *J. spicigera* and *J. torresii*; thecae separation ratio 0.19–0.23). The most highly divergent thecae (i.e. thecae separation ratio 0.58–0.74) are present in *J. comata*, *J. filibracteolata*, *J. kuntzei*, *J. salviiflora*, *J. trichotoma* and *J. vernalis*. Corollas with long tubes and long lower lips (Fig. 6B, C) have evolved at least four times from the optimized ancestral state of corollas with short tubes (<9 mm) and short lower lips (<10 mm).

When examining anther thecae separation and correlated corolla traits with pPCA (Fig. 7), the first two axes of the pPCA explained 82.3 and 10.8 % of the variance. For PC1, corolla tube length and lower lip length loaded positively, in contrast to anther thecae separation, which had high negative loadings (Supplementary Data S5). On PC2, anther thecae separation had high positive loadings. Clusters 1 and 4 were distributed discretely across morphospace, whereas 2 and 3 clustered together in the plot of PC1 and PC2 from the pPCA.

Results from mvMORPH show that OUM models, allowing for separate trait optima for each of the four floral form clusters, were significantly better than OU, BM and BMM models (Supplementary Data S6). All tested traits under the OUM model, both univariate and multivariate, had AICc weights of 1.0 across all trees, indicating that phylogenetic uncertainty was not prevalent in our data. Due to evidence of strong associations between anther thecae separation and corolla tube length and lower lip length, we investigated variation in these traits simultaneously to test for significant shifts in morphology and for convergence. Across the MCC tree (Fig. 8A), under the best-fitting pBIC model (pBIC = 17.33), there were three significant evolutionary shifts of anthers, corolla tube length and lower lip length. This result was also present across the posterior distribution of 100 trees (Supplementary Data S7). With the exception

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TABLE 2. *Results from mclust showing mean character values for each of four clusters. Mean raw values (mm) and standard errors are provided for traits in each cluster group. The VEV model resulted in four clusters. LnL =* 281.96, n = 86, *d.f.* = 71, *BIC* = 247.67. *Total* N = 86, *reflective of three samples per species except* J. concavibracteata, *for which* N = 2. *LLW, lower lip width (mm); LLL, lower lip length (mm).*

	TL	Midpoint	Mouth	LLW	LLL
Cluster 1 (*N* = 30): *J. adenothyrsa, J. calycina, J. colorifera, J. macrantha, J. mendax, J. oerstedii, J. sonorae, J. spicigera, J. tenuistachys, J. torresii*	20.9 (1.3)	3.0 (0.3)	5.1 (0.3)	6.2 (0.9)	15.1 (1.7)
Cluster 2 (*N* = 36): *J. alpina, J. americana, J. boliviana, J. breviflora, J. candelarae, J. filibracteata, J. galapagana, J. kuntzei, J. ovata, J. salviiflora, J. trichotoma, J. vernalis*	6.6 (0.8)	2.4 (0.3)	3.6 (0.4)	7.1 (0.6)	7.2 (1.0)
Cluster 3 (*N* = 14): *J. comata, J. concavibracteata, J. metallica, J. lanceolata, J. pectoralis,*	4.3 (1.1)	1.0 (0.1)	1.9 (1.0)	3.6 (0.7)	4.1 (0.8)
Cluster 4 (*N* = 6): *J. hilsenbeckii, J. ramosa*	16.3 (1.3)	0.9 (0.1)	1.0 (0.1)	9.6 (1.8)	5.9 (0.9)

of the shift involving *J. comata,* which has the smallest corollas of taxa sampled here, these shifts are to corollas with longer floral tubes and lower lips, and to anthers with nearly parallel thecae, and away from the contrasting traits (i.e. shorter floral tubes and lower lips, divergent anther thecae). In the pBIC model, all three significant shifts have moderate to strong bootstrap support, and there was no support for convergence, indicating that these traits are evolving towards three unique optima. In contrast, the best-fitting AICc model (AICc = 11.02) found only two significant shifts; the shift involving *J. comata* was not significant (Fig. 8B; Supplementary Data S7). Unlike the pBIC model, the AICc model detected convergence in these two significant regime shifts, in the clade comprising [(*J. spicigera* + *J. colorifera*) + *J. macrantha*] and *J. calycina*.

DISCUSSION

We took a quantitative morphometric approach to examine variation in floral form among species of the DSP clade of WH Justiciinae. Based on corolla measurements, the mclust analysis identified four relatively discrete clusters across morphospace (Fig. 4). The data further show that morphological variation of anthers among taxa in the DSP clade is correlated with corolla traits. Results indicate that significant correlated evolutionary change in androecial and corolla morphology has occurred across the phylogeny, with multiple shifts to the traits that distinguish cluster 1 (i.e. reduced thecae separation, long corollas). Limited data on pollinator relationships, as well as observations of floral visitors (Supplementary Data S8), suggest that these floral traits are likely to have evolved under selection by pollinating animals (see below).

Cluster 1 includes ten taxa from the DSP clade and is of species with the largest flowers in the clade (Table 2; Fig. 5). Corollas average ~35 mm in total length and have long tubes (~20.9 mm) and lower lips (~15.1 mm). Lower lips lack the herringbone pattern that is common across *Justicia.* This patterning may function as a nectar guide for insects (McDade *et al.*, 2000; Darbyshire *et al.*, 2010; Kiel *et al.*, 2017), as has been proposed or demonstrated by numerous other authors (e.g. Sprengel, 1793; Dafni *et al.*, 1997; Leonard and Papaj, 2011). Corolla colour for taxa in cluster 1 ranges from red to orange to yellow to bright purple. These traits are consistent with hummingbird pollination (Supplementary Data S8), an inference that is supported by published studies (*J. tenuistachys*; Schmidt-Lebuhn *et al.*, 2007), as well as observational evidence [*J. spicigera* (Fig. 2A), C. A. Kiel; *J. adenothyrsa* (Fig. 2G), A. E. Fisher, (Supplementary Data Video S1)]. An exception is *J. sonorae*, which has purple corollas with herringbone-patterned lower lips and narrow tubes, and seems likely to be pollinated by Lepidoptera (Daniel, 2004). Observations suggest a mixed pollination system as hummingbirds, hawkmoths and butterflies have been observed to visit flowers of *J. sonorae* at the Arizona-Sonora Desert Museum (https://www.desertmuseum.org/visit/sheets/Jusson.pdf).

Cluster 2 is of taxa with relatively short corollas (~14 mm on average) and corolla tubes (~6.6 mm), relatively broad lower lips (~7.1 mm) and openings at the mouth (~3.6 mm) (Table 2; Fig. 5), and floral traits consistent with bee pollination (Supplementary Data S8). A few species in this group have

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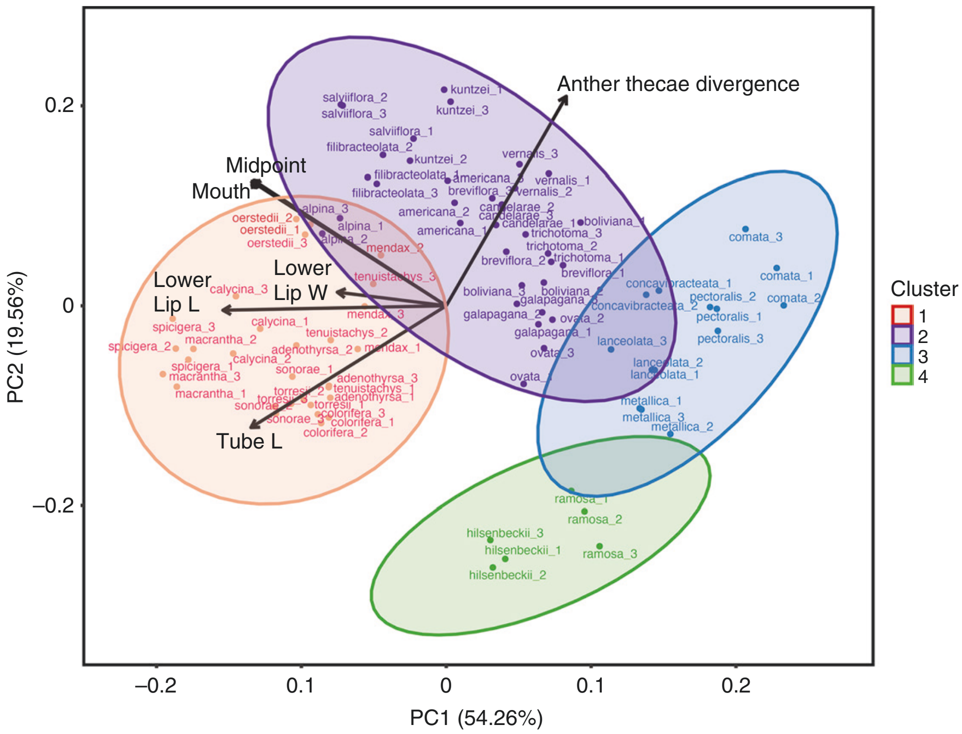

FIG. 4. PCA of corolla and anther traits in the DSP clade; the first two PCs explain 54.3 and 19.6 % of the variance, respectively. Species names are associated with the three points representing samples (only two for *J. concavibracteata*). Colour groupings represent cluster assignments from the mclust analysis. Cluster 1 is of plants with traits consistent with hummingbird pollination; clusters 2 and 3 are consistent with bee/fly pollination; cluster 4 is consistent with lepidopteran pollination.

flowers with hooded upper lips [i.e. *J. breviflora* (Fig. 2D) and *J. salviiflora* (Fig. 2C)]. Corollas range in colour from white to lavender to bright purple. Unlike group 1, all have conspicuous herringbone markings on the surface of the lower lip that contrast markedly in colour with the corolla; in some cases, the markings are elevated above the surface of the lower lip. These traits point to pollination by insects; available data and observations support this hypothesis. Honeybees (*Apis mellifera*, Apidae) have been observed as frequent visitors to *J. salviiflora* in Mexico (Fig. 2C; Supplementary Data Video S2) and are also documented as the primary pollinators of *J. kuntzei* in Bolivia (Schmidt-Lebuhn *et al*., 2019). Stingless bees, *Trigona* spp. (Apidae), which rob nectar and pollen from flowers of many plant species, were observed frequently visiting flowers of *J. filibracteolata* in Santa Maria, Colombia [C. A. Kiel and P. Saab pers. obs. (Supplementary Data Video S3)]. During these visits, bees had extended contact with the stigma and anthers, and pollen was deposited on the animals' dorsal and lateral surfaces, consistent with the hypothesis that *Trigona* bees are effective pollinators of *J. filibracteolata*. For *J. galapagana*, the carpenter bee *Xylocopa darwini* (Apidae) and the bee-mimicking fly *Toxomerus crockeri* (Diptera: Syrphidae) are documented as effective pollinators (McMullen, 1994). Flowers of *J. americana* are visited by a variety of insects including bees, flies and butterflies (Supplementary Data S8).

Cluster 3 is composed of taxa with the smallest flowers in the DSP clade (mean of ~8.5 mm in length), with lower lip width and length of ~3.6 and ~4.1 mm, respectively (Table 2; Fig. 5). As for cluster 2, flowers of all cluster 3 species have herringbone markings on the lower lip. Little is known about pollinators or floral visitors of species in this group (Supplementary Data S8). *Justicia pectoralis* is likely pollinated by 'small insects' based on observations by Croat (1978) and *Trigona* bees have been frequently observed visitors (Belz and Wang, 2018; Rocha, 2020; A. E. Fisher, pers. obs.). Schmidt-Lebuhn *et al*. (2019) documented large and small stingless bees (*Trigona* spp.) as effective pollinators of *J. comata* (Fig. 2I) in Bolivia. These authors also note that *J. comata* is visited by lepidopterans, although at a significantly lower rate than by *Trigona* spp. (40 versus 132 visits). Schmidt-Lebuhn *et al*. (2019) note that it was unclear whether the lepidopterans effectively interacted with the plants' reproductive organs.

Cluster 4 is of two species with corollas that are ~24 mm long, with narrow floral tubes (~1 mm wide) and ~16 mm long (Table 2; Fig. 5). Upper lips are short and narrow, and lower lips are reflexed and broad (~9.5 mm). Hilsenbeck (1990) and Daniel (2004) have suggested that Lepidoptera are likely the effective pollinators of *J. hilsenbeckii* (Fig. 2G) and *J. ramosa* (Fig. 2B; Supplementary Data S8).

Our results also reveal intriguing patterns of variation in anther morphology across the discrete clusters. Clusters 2 and 3 have anther traits that are not distinguishable from one another; both have thecae that are strongly divergent (Fig. 5). As just discussed, evidence indicates that insects, particularly bees and flies, are the primary visitors to flowers in both clusters, pointing to a relationship between anthers with strongly separated thecae and insect pollination in the DSP clade. The

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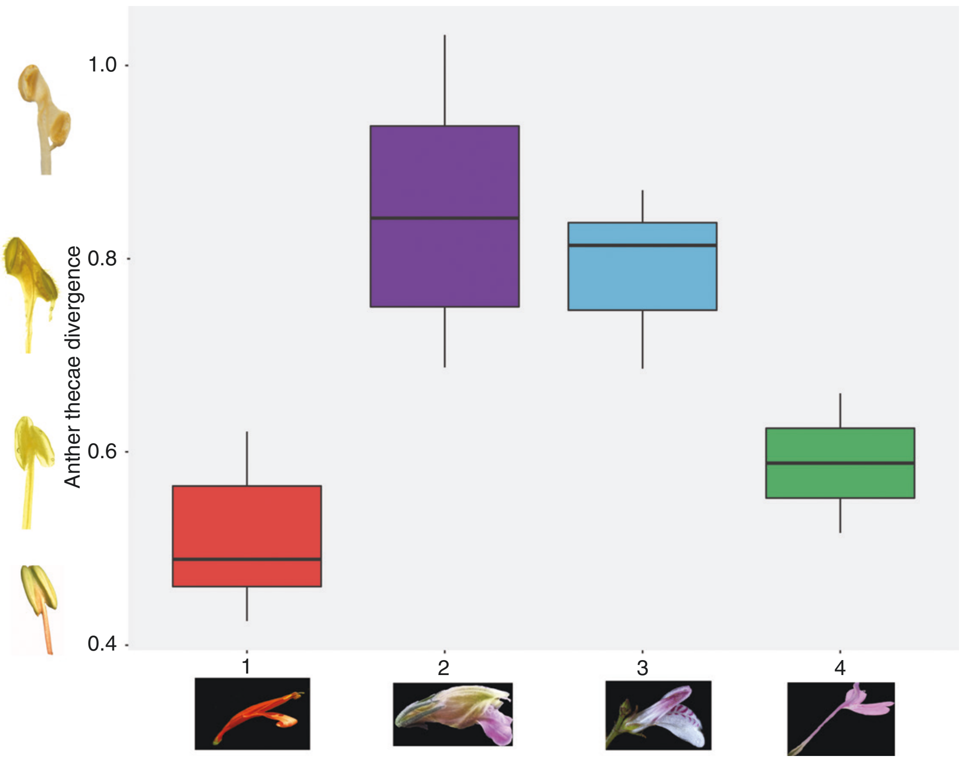

FIG. 5. Box and whisker plot displaying median, upper and lower quartiles and minimum and maximum values for transformed thecae separation ratios among the four cluster groups from the mclust analysis (i.e. floral form types) in the DSP clade. Cluster 1 is of plants with traits consistent with hummingbird pollination; clusters 2 and 3 are consistent with bee/fly pollination; cluster 4 is consistent with lepidopteran pollination. Illustrations on the *x* and *y* axes are of representative corolla and anther morphologies for each cluster group.

TABLE 3. *Phylogenetic ANOVA:* post hoc *test on means of anther thecae separation between floral cluster groupings. Cluster 1 is of plants with traits consistent with hummingbird pollination; clusters 2 and 3 are consistent with bee/fly pollination; cluster 4 is consistent with lepidopteran pollination. Sum square = 0.69, mean square = 0.23,* F *= 23.98,* P *= 0.001.* *P *< 0.05.*

	Cluster 1		Cluster 2		Cluster 3		Cluster 4	
	t	P	t	P	t	P	t	P
Cluster 1	0.00	1.00	−8.07	0.006*	−5.20	0.006*	−1.01	0.52
Cluster 2			0.00	1.00	1.14	0.52	3.50	0.04*
Cluster 3					0.00	1.00	2.47	0.17
Cluster 4							0.00	1.00

cluster analysis may distinguish these two groups based on flower size: those of plants in cluster 2 are almost twice as large as those in cluster 3. We predict that flowers of plants in these two clusters are pollinated by insects of different sizes. Species in cluster 1, with corolla morphology consistent with hummingbird pollination, have anthers that differ significantly from those of plants in clusters 2 and 3 (Table 3), but not cluster 4. Taxa placed in clusters 1 and 4 have anther thecae that are nearly parallel to slightly divergent (Fig. 5). Ancestral state optimization indicates that these traits are likely derived across the DSP lineage, with reduced thecae separation accompanying the changes in corolla size and form that characterize clusters 1 and 4. It is noteworthy that anther traits are similar in plants likely pollinated by hummingbirds (cluster 1) and Lepidoptera (cluster 4).

Correlations of corolla traits and thecae separation from PGLS analysis provide further support for the PCA results (Supplementary Data Fig. S3). The PGLS results, in particular, reveal that length rather than width of the corolla is associated with the evolution of androecial form (Table 5). Overall, our results point to correlated changes in suites of floral traits, particularly corolla tube length, lower lip length and anther thecae separation. In turn, multiple lines of evidence are consistent with the hypothesis that these correlated changes are associated with shifts in pollinators and can be hypothesized to represent adaptive evolution.

Quantitative analyses indicated that most of the individual traits that we measured are not phylogenetically constrained. It is especially interesting that thecae separation is not phylogenetically patterned (Figs 6 and 7), suggesting that this trait

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has been evolutionarily vagile. This hypothesis is supported by results from Blomberg's *K*: for many traits, *K* is not statistically different from 0, meaning that their distribution cannot be explained by phylogenetic history alone (Table 4). We expected that corolla tube length would be at least as evolutionarily vagile as thecae separation because PGLS showed a strong correlation between the two traits (Supplementary Data Fig. 3). However, Blomberg's *K* indicates that there is some degree of phylogenetic pattern across the DSP clade with regard to corolla tube length (i.e. *K* is significantly different from 0). We suspect that two clades, one of *J. spicigera*, *J. macrantha* and *J. colorifera* and the other of *J. ramosa*, *J. hilsenbeckii* and *J. sonorae*, all species with relatively long corolla tubes, are the source of the phylogenetic signal for this trait (Fig. 6). We predict that, with a larger sample size across WH Justiciinae, *K* would be indistinguishable from 0 for corolla tube length.

The best-fitting OUM model from mvMORPH, which incorporates cluster groups into the model, suggests that these floral traits likely evolved under selection (Supplementary Data S6). These results provide additional support for the hypothesis that anther thecae separation and corolla traits are correlated, and correspond to particular pollination systems. Further support comes from results of the l1ou analyses, which tested anther thecae separation, corolla tube length and lower lip length together as a suite (Fig. 8). Shifts detected in the l1ou analyses under the pBIC and AICc models primarily corresponded to taxa of cluster 1, plants likely hummingbird pollinated (Supplementary Data S7). While the pBIC did not detect convergence, the AICc model (Fig. 8B) revealed two shifts as convergent, indicating that [(*J. spicigera* + *J. colorifera*) + *J. macrantha*] and *J. calycina*, all species with corolla traits consistent with hummingbird pollination, evolved towards a similar trait optimum. We posit that with increased sample size to include all ten major lineages of WH Justiciinae, more instances of convergence will likely be revealed as significant. Further, it is intriguing that our analysis primarily identifies shifts to traits associated with hummingbird pollination as significant (with the exception of *J. comata*). This may reflect the diversity of animals that likely pollinate flowers of the other species in this clade. Alternatively, more complete sampling may reveal other significant patterns of shifts. It is also possible that additional traits would need to be measured (e.g. relative orientation of the upper and lower lips) to tease out patterns associated with multiple guilds of insect pollinators.

TABLE 4. *Blomberg's* K *values for traits across the MCC tree followed, in parentheses, by values across 100 trees sampled from the posterior distribution (median parameter of distribution; minimum, maximum values).* K *values significantly different from* K = *0 are bold.*

Trait	**Blomberg's K**	**P $_{(K = 0)}$**
Thecae separation ratio	0.73 (0.69; 0.34, 0.89)	0.07 (0.07; 007, 0.51)
Tube length	0.83 (0.80; 0.64, 0.98)	**0.02** (0.02; 0.001, 0.12)
Tube midpoint width	0.54 (0.52; 0.27, 0.65)	0.44 (0.43; 0.17, 0.66)
Corolla mouth opening width	0.44 (0.41; 0.22, 0.56)	0.82 (0.81; 0.53, 0.98)
Lower lip width	0.67 (0.66; 0.12, 0.80)	0.10 (0.11; 0.001, 0.34)
Lower lip length	0.63 (0.59; 0.30, 0.75)	0.18 (0.20; 0.07, 0.55)

Within this relatively small clade of WH Justiciinae, floral form consistent with hummingbird pollination has evolved at least four times from the putative ancestral condition of bee/fly pollination, each time accompanied by transitions to anther thecae that are more or less parallel rather than markedly separated (Fig. 6). In contrast, across the DSP lineage, taxa with short corolla tubes and lower lips (i.e. floral form consistent with bee/fly pollination) have thecae with the greatest separation. This is supported by the PCA that includes all floral and anther traits, and demonstrates an inverse relationship between thecae separation and corolla tube length and lower lip length

TABLE 5. *Correlation tests between corolla traits and thecae separation ratio from PGLS under the OU model. Parameter estimates for PGLS analyses for the MCC tree (unshaded) and across 100 tree sampled from the posterior distribution (shaded), median parameter values (minimum, maximum), α, selective strength and σ^2, rate of stochastic motion. Significantly correlated traits are bold.*

Model	**α**	**Coefficient**	**SE**	***P***	**σ^2**
Tube length ~ thecae	0.23	–0.36	0.07	**0.0001**	0.24
	0.25 (0.14, 5.9)	–0.37 (-0.39, -0.36)	0.08 (0.075, 0.079)	**< 0.0001** (<0.0001, <0.0001)	0.13 (0.13, 0.14)
Tube midpoint width ~ thecae	0.20	–0.16	0.13	0.25	0.17
	0.22 (0.11, 12.83)	–0.15 (-0.18, -0.12)	0.13 (0.12, 0.14)	0.27 (0.18, 0.36)	0.18 (0.17, 0.18)
Corolla mouth opening width ~ thecae	0.17	–0.21	0.13	0.12	0.17
	0.18 (0.09, 4.49)	–0.21 (-0.25, -0.15)	0.13 (0.10, 0.15)	0.13 (0.07, 0.18)	0.17 (0.17, 0.18)
Lower lip width ~ thecae	0.02	0.02	0.17	0.94	0.91
	0.20 (0.10, 13.12)	0.02 (–0.03, 0.06)	0.18 (0.17-0.19)	0.90 (0.75, 1.0)	0.18 (0.18, 0.19)
Lower lip length ~ thecae	0.34	–0.34	0.11	**0.005**	0.22
	0.23 (0.12, 6.67)	–0.34 (-0.37, -0.30)	0.11 (0.10, 0.12)	**0.005** (0.003, 0.01)	0.15 (0.15, 0.16)

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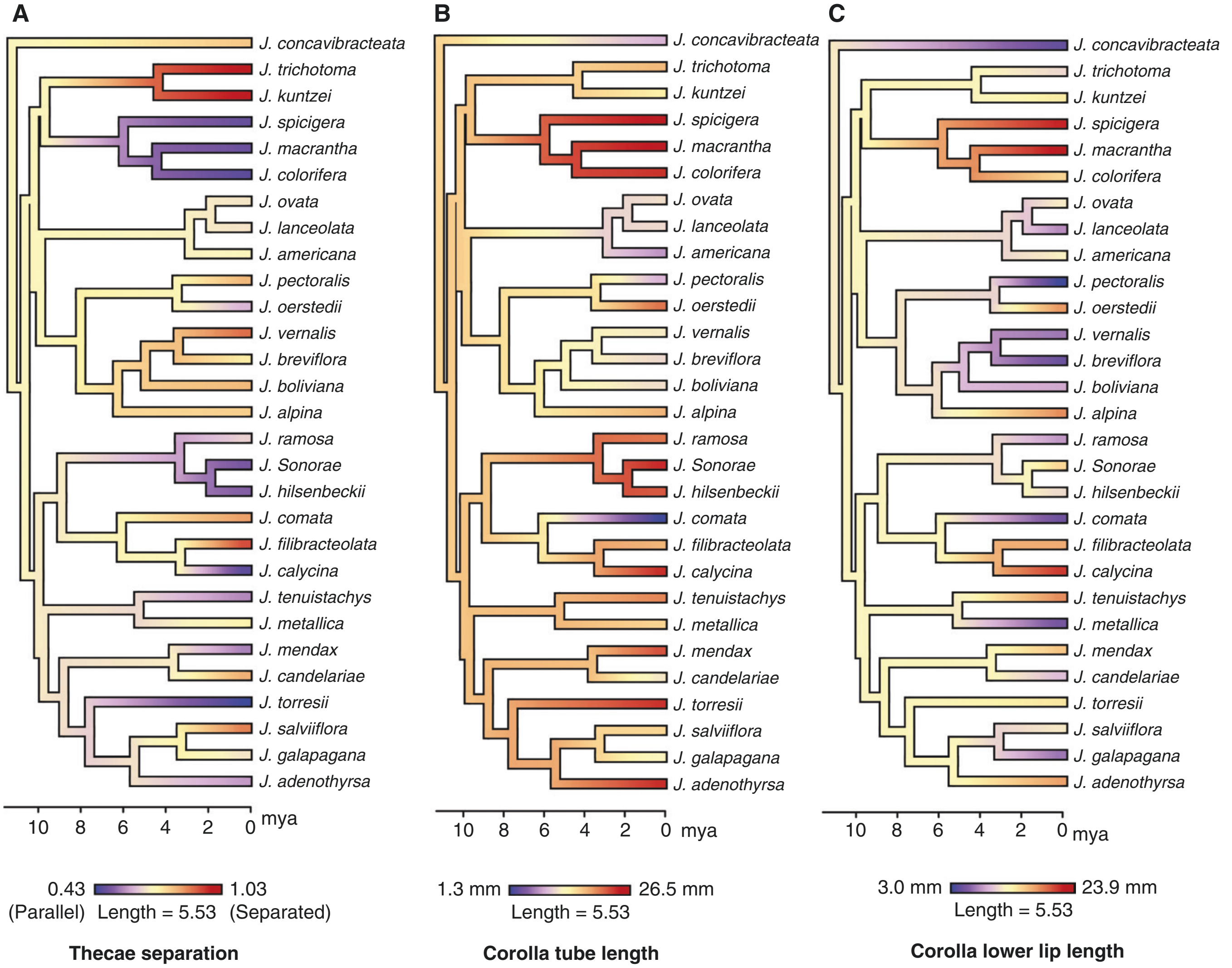

Fig. 6. Ancestral state reconstruction along the nodes and branches of the DSP clade illustrating (A) thecae separation, (B) corolla tube length and (C) corolla lower lip length. Deeper red colours represent species that have anthers with a greater degree of separation (A) and those with longer corolla tubes (B) and longer lower lip lengths (C). Raw values were plotted using the *contMap* function in phytools.

(Fig. 4). Specifically, as thecae separation increases, corolla tube and lower lip length decrease.

Shifts in floral morphological traits are common in other major clades of Acanthaceae. For example, in the *Tetramerium* lineage (Justicieae), a group of 170 species that exhibits notable floral diversity, particularly among WH species, corolla traits consistent with hummingbird pollination have evolved at least eight times (McDade *et al.*, 2018). In *Ruellia*, where floral form and colour are exceptionally labile, there is evidence of multiple transitions from hummingbird pollination to bee or generalized insect pollination (Tripp and Manos, 2008; Tripp and Tsai, 2017). Beyond Acanthaceae, similar patterns of floral traits and pollinator shifts have been reported in other angiosperm groups (e.g. Marten-Rodriguez, 2010; Wessinger *et al.*, 2016; Lagomarsino *et al.*, 2017; Joly *et al.*, 2018; Smith and Kriebel, 2018). In contrast, few studies have examined androecial morphology in an evolutionary context, much less in the context of changes in floral size and form (but see Kriebel *et al.*, 2020). In *Salvia*, corolla form, anther connective shape, and style shape are correlated with bee and hummingbird pollination (Kriebel *et al.*, 2020). In contrast with the present study, Kriebel *et al.* (2020) did not examine the pollen-producing anthers; instead, these authors studied evolution of the shape of the connective tissue that yields the functional lever mechanism. Within the DSP lineage, experimental studies of how anthers with different degrees of thecae separation function in pollination are lacking. Likewise, whether or not anther appendages function in pollination is not known in *Justicia.* It has been hypothesized in other angiosperm groups that secondary androecial structures or floral appendages such as trichomes may act as pollen scrapers to remove heterospecific pollen from the floral visitor's body prior to placement of conspecific pollen (Minnar *et al.*, 2019). For example, field observations of flower visitors in a few species offer insights that provide context for studies using experimental approaches. In *J. galapagana*, the bee *Xylocopa darwini* and the fly *Toxomerus crockeri* were seen to push their way into the corolla throats (McMullen, 1994). This movement caused the insect's dorsal surface to rub against

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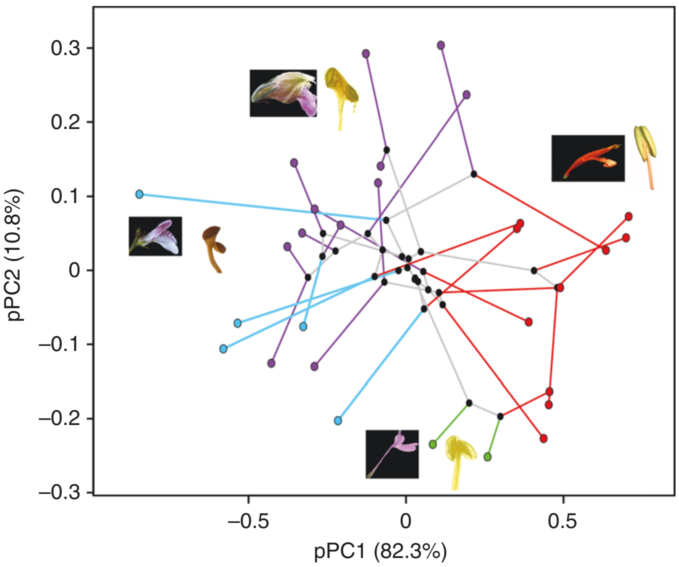

FIG. 7. pPCA of corolla traits correlated with anther thecae separation (tube length, lower lip length) from 29 species in the DSP clade using the MCC tree topology. The first two PC axes account for 93.1 % of the variance. Cluster groups 1–4 from the mclust analysis are depicted by coloured branches and terminals: cluster 1 (red) is of plants with traits consistent with hummingbird pollination; clusters 2 and 3 (purple, blue respectively) are consistent with bee/fly pollination; cluster 4 (green) is consistent with lepidopteran pollination. Examples of corolla and anther morphology for each cluster group are given. Photographs by C. Kiel.

the flower's anthers and stigma. Croat (1978) observed small insects crawling beneath the arched stamens of flowers of *J. pectoralis*, spreading pollen on the insects' dorsal surfaces. At the same time, the style was released from the stylar furrow (i.e. rugula: a channel-like structure in the upper lip of the corolla that partially encloses the style in these plants), striking the visiting animals' dorsal surface.

Our field observations of *J. salviiflora* (group 2), a species with strongly divergent anther thecae, point to a dynamic interaction between flowers and insect visitors. The androecium conforms to the back and sides of a visiting bee as it enters the mouth and throat of the corolla, seeming to clamp onto the bee's body. As the bee exits the corolla, the anthers de-clamp from the animal's body (A. E. Fisher and C. A. Kiel, pers. obs., Supplementary Data Video S2). We hypothesize that this mechanism deposits pollen on bees' bodies in locations where it cannot be reached by the grooming bee. This would have the effect of placement that yields both effective transfer to stigmas and pollen carry-over to flowers beyond the first visited after the pollen is deposited. An increase in male fitness would select for androecial morphologies that function most effectively in this way. We further speculate that divergent thecae may be non-functional and even maladaptive in flowers that are pollinated by Lepidoptera or hummingbirds. Clearly, detailed experimental studies of androecial function and pollination in this group will provide further insight into the evolution of androecial form.

In contrast, in hummingbird- and lepidopteran-pollinated *Justicia*, the intimate animal:plant interface at the time of pollination apparently functions differently. In the case of bird- and lepidopteran-pollinated flowers, the often exserted stamens with parallel thecae are held closely parallel to the upper lip of the corolla near the stigma, which, along with the style, is held in place by the rugula. These structures may function in tandem to result in effective placement of pollen on the bird's head or back, and transfer of pollen to the stigma. Parallel (or nearly so) thecae are not common among EH *Justicia*, where hummingbirds do not occur. Interestingly, among EH Justiciinae that do exhibit anthers with nearly parallel thecae are species of *Anisotes* (Kiel *et al*., 2017). Historically, taxa have been placed in *Anisotes* primarily based on floral traits: large, colourful flowers (red to red-orange to pink to yellow-green) with corolla lips nearly twice as long as the tube. These species are either known to be pollinated by passerine birds or have floral form consistent with pollination by these birds (Kiel *et al*., 2017; Manzitto-Tripp *et al*., 2022). Like many other genera in Acanthaceae that have been based on floral morphology, *Anisotes* is not monophyletic; instead, this corolla morphology, along with parallel anther thecae, is hypothesized to have evolved at least five times (Kiel *et al*., 2017).

Multiple taxa of lepidopteran visitors have been observed among species of *Justicia*, with the phenology of corolla anthesis corresponding to animal activity patterns (e.g. diurnal butterflies versus nocturnal hawkmoths (Hilsenbeck, 1990; Aizen and Feinsinger, 1994; Rodriguez, 2022). For lepidopteran-pollinated species in WH *Justicia*, effective pollen placement on the insect's proboscis may happen in many ways and is apparently not as geometrically guided as pollen placement on bees or flies. We hypothesize that the narrow corolla tube serves to position flower visitors for pollen transfer such that there is no functional necessity for highly displaced thecae. Both narrow and long corolla tubes have been associated with effective pollen transfer and pollination efficiency in lepidopteran-pollinated plants (Campbell *et al*., 1997; Alexandersson and Johnson, 2002; Kaczorowski *et al*., 2012). Although many lepidopterans groom pollen from their proboscis (Hlavac, 1975; Hikl and Krenn, 2011), this behaviour is spatially generalized such that there would not be selection for precise placement of pollen. This idea is supported by floral morphology associated with lepidopteran pollination in *Justicia*: corollas are narrow and anthers are usually located near the throat of the corolla, where they seem to deposit pollen passively as the probing proboscides pass into the corolla tube.

In summary, we took a multivariate approach to place species in the DSP clade into four distinct clusters based on corolla traits. These traits (along with others that were not part of the model) are predicted or known to be associated with pollination by hummingbirds, lepidopterans and large to small bees. To this, we have added the entirely novel result that there is startling variation in androecial traits among species in this relatively small clade, and that this variation is strongly associated with variation in floral form. Whereas species that are known or likely to be hummingbird pollinated have stamens with parallel, largely unornamented thecae, those that are likely bee pollinated have stamens with offset, divergent thecae that tend to have appendages of various sorts. We have also shown that there is remarkable evolutionary vagility in these traits across the DSP clade with little signal of phylogenetic constraint. Our results suggest that thecae separation is likely under selection in concert with a suite of other floral characters.

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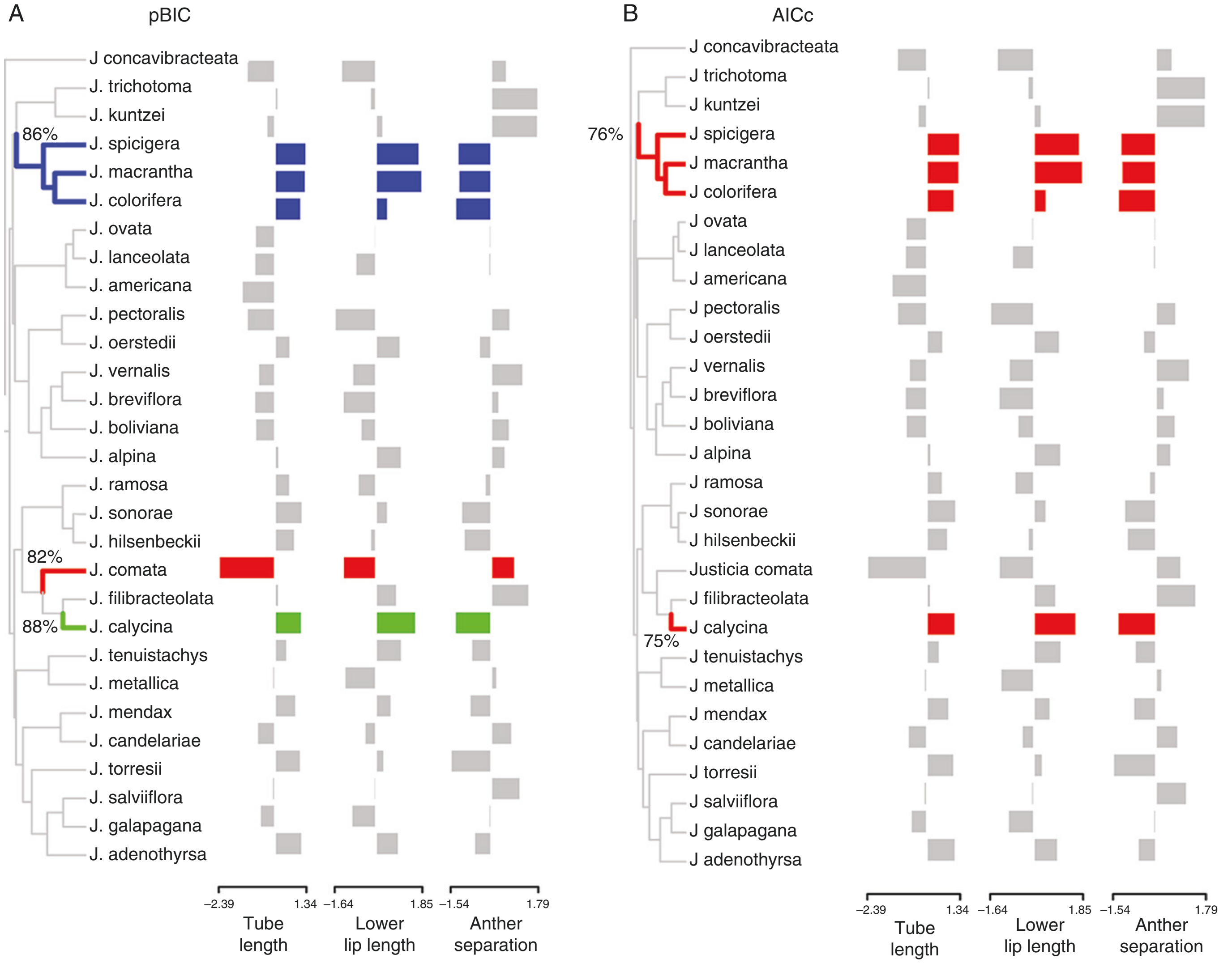

Fig. 8. Shift detection analysis with l1ou for anther thecae separation, corolla tube length and lower lip length among *Justicia* from the DSP clade using the MCC tree. The barplots illustrate the transformed mean values for each trait. Coloured lines separate regimes for the best shift configuration from the (A) pBIC model selection criterion and (B) AICc model selection criterion. Similar colours indicate convergent regimes.

As has been shown by others (Armbruster, 1991; Armbruster *et al.*, 2004; Pérez Barrales *et al.*, 2007; Rosas-Guerrero *et al.*, 2011; Kriebel *et al.*, 2020), our results support the hypothesis that floral structures function in an integrated manner and are likely subject to selection as a suite. This may happen when a change in one character results in subsequent changes in one or more other characters due to linked or constrained genetic or developmental processes. Alternatively, several characters could be responding independently to selection. We hypothesize that similar patterns of anther thecae separation will be documented in other major WH Justiciinae lineages that exhibit similar diversity in floral form. Other floral traits related to pollen placement efficiency (e.g. anther heights, stigma position and shape) may show similar patterns of covariation in Justiciinae. Variation in traits associated with pollen placement may be more important in influencing overall plant fitness than floral traits related to visitor attraction (Rosas-Guerrero *et al.*, 2011). For example, floral traits such as colour may attract both pollinators and floral antagonists, which may cause opposing selection on floral display (Strauss and Whittall, 2006).

Pollinator-mediated selection on correlated traits (Fenster *et al.*, 2004; Temeles *et al.*, 2013; Lagomarsino *et al.*, 2017; Smith and Kriebel, 2018) is not without debate and several studies have failed to support this hypothesis (Herrera, 2001; Herrera *et al.*, 2002; Bissel and Diggle, 2008). It is possible that patterns of phenotypic correlations may be caused by pleiotropy or linkage disequilibrium. Clearly, in-depth field and laboratory studies of the interface between pollinators and the flowers that they visit (with an emphasis on anthers and stigmas) are vital to understand the selective forces behind the correlative patterns that we show here. The clade focused on here would be ideal for such studies given the diversity encompassed and the fact that many of these plants are easily cultivated. We hypothesize that selection on floral traits by pollinators has been a major force driving the remarkable species richness of the WH Justiciinae clade. We intend to expand the present sample to include many

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more taxa, and to extend our study to include other traits, such as pollen and gynoecial structures. These are clearly as critical as anthers to the plant–pollinator interface and amply merit research.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Data S1: voucher information for taxa along with corolla trait measurements. Data S2: comparison of floral measurements from different preservation methods. Data S3: results for the first three principal components of the PCA of six traits. Data S4: results of the one-way ANOVA and Tukey's HSD *post hoc* test. Data S5: loadings for each trait for pPC1, pPC2 and pPC3 axes from the pPCA. Data S6: results from mvMorph. Data S7: results from the l1ou analyses for 100 trees across the Bayesian posterior distribution. Data S8: Pollinator data, floral visitor observations, and pollinator inferences based on trait data for species lacking pollinator data or observations. Where pollinator data are not available, pollination syndromes (sensu Faegri and van der Pijl, 1979; Fenster *et al*., 2004; Rosas-Guerrero *et al*., 2014; Lagomarsino *et al*., 2017) have been found to be useful (but see Waser *et al*., 1996; Ollerton *et al*., 2009; Duffy *et al*., 2020). Figure S1: MCC tree from the BEAST analysis for the Justiciinae. Figure S2: MCC tree from the BEAST analysis explaining details of relationships among members of the DSP clade with time scale below. Figure S3: phylogenetic generalized least squares regression under the OU model depicting correlations between anther thecae separation and corolla tube length and between anther thecae separation and corolla lower lip length for 29 species in the DSP clade. Video S1: flowers of *J. adenothyrsa* visited by *Archilochus* sp. in Guerrero, Mexico; video by A. E. Fisher. Video S2: *Apis mellifera* visiting flowers of *J. salviiflora* in Guerrero, Mexico; video by A. E. Fisher. Video S3: *Trigona* spp. visiting flowers of *J. filibracteolata* in Santa Maria, Colombia; video by C. A. Kiel.

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