








Pollinator shifts, contingent evolution, and evolutionary constraint drive floral disparity in *Salvia* (Lamiaceae): Evidence from morphometrics and phylogenetic comparative methods

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Switches in pollinators have been argued to be key drivers of floral evolution in angiosperms. However, few studies have tested the relationship between floral shape evolution and switches in pollination in large clades. In concert with a dated phylogeny, we present a morphometric analysis of corolla, anther connective, and style shape across 44% of nearly 1000 species of *Salvia* (Lamiaceae) and test four hypotheses of floral evolution. We demonstrate that floral morphospace of New World (NW) *Salvia* is largely distinct from that of Old World (OW) *Salvia* and that these differences are pollinator driven; shifts in floral morphology sometimes mirror shifts in pollinators; anther connectives (key constituents of the *Salvia* staminal lever) and styles co-evolved from curved to linear shapes following shifts from bee to bird pollination; and morphological differences between NW and OW bee flowers are partly the legacy of constraints imposed by an earlier shift to bird pollination in the NW. The distinctive staminal lever in *Salvia* is a morphologically diverse structure that has evolved in concert with both the corolla and style, under different pollinator pressures, and in contingent fashion.

KEY WORDS: Bee pollination, bird pollination, floral shape, key innovation, staminal lever.

A major challenge of evolutionary biology is clarifying factors that contribute to morphological variation among species. In angiosperms, the remarkable diversity of floral form and size points to the fundamental role and power of natural selection in shaping this diversity. The pressures exerted by animal pollination are often invoked as causal for much of this floral diversity (Fenster et al. 2004; Armbruster 2014; Gómez et al. 2015) and species

diversification (Forest et al. 2014; Breitkopf et al. 2015). Phylogenetic comparative methods (PCMs) that examine the evolution of quantitative morphological traits under specific ecological selective regimes can be applied to answer major questions such as how pollinators impact the evolution of floral form and size (e.g., Smith 2010; Lagomarsino et al. 2017; Joly et al. 2018; Smith and Kriebel 2018). Assessing the impact of earlier

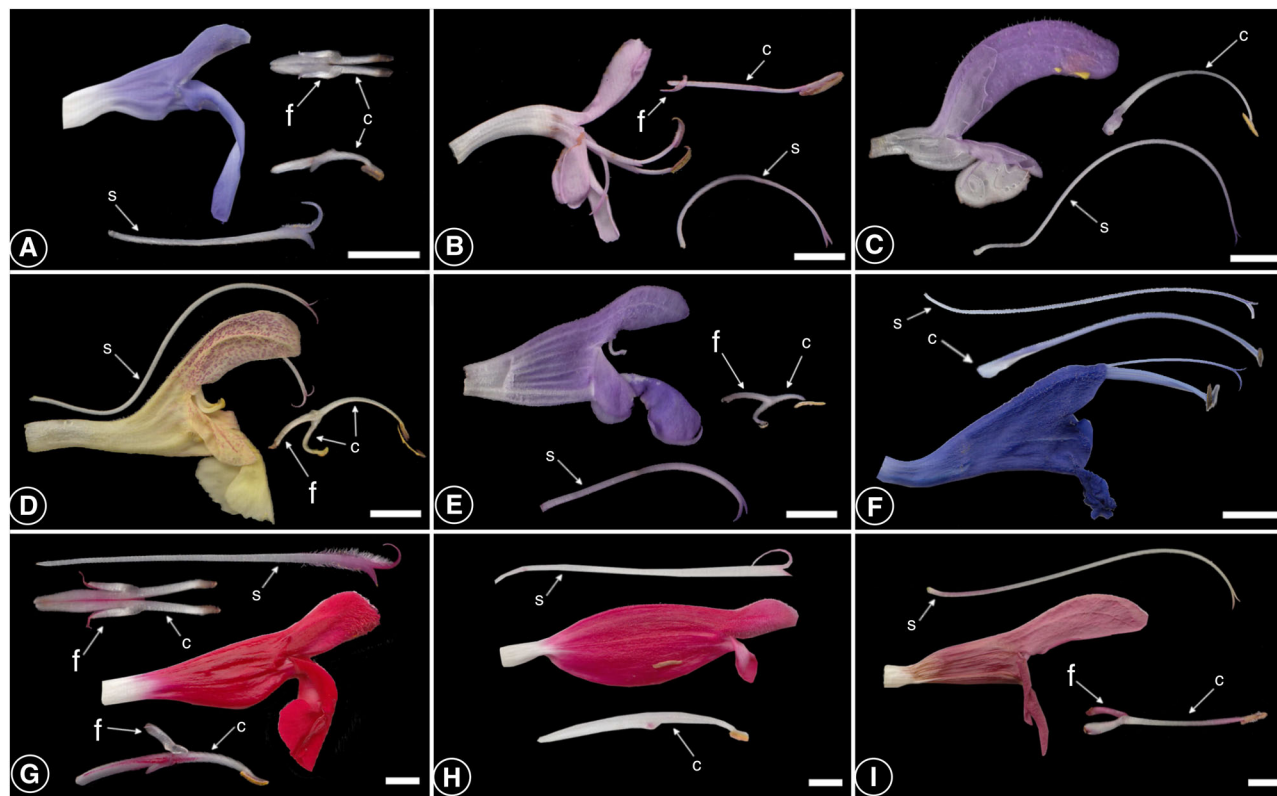


Figure 1. Representative diversity of *Salvia* flowers (corollas, anther connectives, and styles in side view) with different pollinators and from different biogeographic areas. Subgeneric names follow Kriebel et al. (2019). Bee, New World: A, *Salvia chamaedryoides* (subg. *Calosphace*); B, *S. leucophylla* (subg. *Audibertia*). Bee, Old World: C, *S. chrysophylla* (subg. *Sclarea*); D, *S. nubicola* (subg. *Glutinaria*); E, *S. officinalis* (subg. *Salvia*). Bird, New World: F, *Salvia sagittata* (subg. *Calosphace*); G, *S. greggii* (subg. *Calosphace*); H, *S. wagneriana* (subg. *Calosphace*). Bird, Old World: I, *S. lanceolata* (subg. “*Heterosphace*”). Panels A and G show an additional view of the anther connective from above to highlight the fusion of their dorsal arms. Acronyms: c = connective; f = filament; s = style.

pollinator-mediated changes in floral form on the evolution of subsequent forms should be possible with this combined phylogenetic and morphometric approach. Such an approach, for example, may provide evidence that the rise of a pollinator-mediated floral novelty has allowed the later origin of specific floral changes (contingent evolution—i.e., “contingent upon” sensu Beatty 2006, Blount et al. 2018). Likewise, this approach may provide support that this earlier novelty has restrained the diversity of subsequent floral changes (evolutionary constraint, sensu Stebbins 1974).

The genus *Salvia* (Lamiaceae), with a worldwide distribution of nearly 1000 species and presence in multiple biomes, is proposed as a model for addressing questions regarding how floral morphology evolves as a result of switches in pollinators. *Salvia* exhibits striking floral variation in terms of corolla, anther connective, and stylar morphology, making it an ideal group to test hypotheses regarding the evolution of flower shape divergence in a worldwide context (Fig. 1). A unique feature of most *Salvia* flowers is the staminal lever (Claßen-Bockhoff et al. 2003, 2004a; Walker and Sytsma 2007; Westerkamp and Claßen-

Bockhoff 2007; Zhang et al. 2011), a feature that promotes pollen transfer and hence outcrossing and that has been posited to be a key innovation for adaptive radiation within *Salvia* (Claßen-Bockhoff et al. 2004a). The staminal lever is a complex structure comprising (in its most elaborate form) two interacting stamens with short filaments, an elongated connective separating the anterior and posterior anther thecae, loss of posterior thecae fertility, and fusion of the two posterior thecae into a paddle that usually blocks the corolla tube and that pollinators must push against (and while doing so get dusted with pollen) to access nectar (Claßen-Bockhoff et al. 2004b; Celep et al. 2020). These staminal levers have developed directly from elongation of the anther connective, but the shape and evolution of the anther connective have not been evaluated using morphometric approaches.

The current phylogenetic framework of *Salvia* includes more than half of the genus and is based on Anchored Hybrid Enrichment (AHE) data coupled with targeted sequencing (Kriebel et al. 2019). The biogeographic history of *Salvia* indicates that it originated in southwest Asia during the Oligocene and later dispersed at least twice independently from the Old World (OW)

to the New World (NW) during the Miocene, several times to the Mediterranean region, twice to South Africa and Madagascar, and twice to east Asia (Will and Claßen-Bockhoff 2017; Kriebel et al. 2019; Hu et al. 2020). Diversification analyses using BAMM version 2.5 (Rabosky et al. 2014) identified four significant shifts in species diversification within *Salvia* (Kriebel et al. 2019). None of these diversification shifts, however, appear to be correlated with shifts in areas or biomes. One diversification shift does precede a change from ancestral bee pollination to hummingbird pollination around 20 million years ago at the crown of NW subg. *Calosphace*, but reversals back to bee pollination occurred repeatedly within the group (Kriebel et al. 2019). The lack of tight correlation of either area, biome, or pollinator shifts to all four shifts in species diversification points to additional drivers of speciation in *Salvia*, including perhaps the interplay of floral features and pollinators.

The study of pollination biology in *Salvia* has a rich history involving case studies of hybridization (Epling 1947; Anderson and Anderson 1954), speciation through reproductive isolation (Grant and Grant 1964; Grant 1994), and two hypothesized key innovations associated with bee pollination: the bilobed corolla (Westerkamp and Claßen-Bockhoff 2007) and the staminal lever mechanism (Sprengel 1793; Hildebrand 1865; Claßen-Bockhoff et al. 2003, 2004a). *Salvia*, primarily a melittophilous genus, has served as an exemplar group for pollination studies of bilabiate flowers by bees (Faegri and van der Pijl 1966; Proctor and Yeo 1973; Westerkamp and Claßen-Bockhoff 2007). Although mixed bee/fly pollination has been documented to increase pollination success in some OW *Salvia*, bees are considered the most efficient pollinator (Celep et al. 2014, 2020). In addition to bees, birds are the other major pollinators of *Salvia* (Wester and Claßen-Bockhoff 2006b, 2007). Hummingbird pollination has been documented in 184 NW species (Trelease 1881; McGregor 1899; Wester and Claßen-Bockhoff 2006a, 2007, 2011). Conversely, bird pollination in the OW has only been documented in three South African species and predicted in a fourth species in Madagascar (Wester and Claßen-Bockhoff 2006b, 2007).

The wealth of information on pollinators across the biogeographical distribution and phylogenetic breadth of *Salvia* prompted several attempts to infer the number of shifts to bird pollination in the large NW subg. *Calosphace*, which includes about 98% of the bird-pollinated species. Subgenus *Calosphace* contains about 560 species and is diverse in terms of floral morphology (González-Gallegos et al. 2020). Most species within the subgenus possess straight anthers and styles, features that are uncommon in OW *Salvia*. Benítez-Vieyra et al. (2014, 2019) included 53 species of subg. *Calosphace* and detected four origins of bird pollination and three reversals back to bee pollination. Fragoso-Martínez et al. (2018), reconstructing bee pollination as ancestral within subg. *Calosphace*, included 247 (~44%) species

of the subgenus and found 13 origins of bird pollination within subg. *Calosphace* and one reversal back to bee pollination. A third study included 519 species of *Salvia* coded for pollinator, with 266 species from subg. *Calosphace* (Kriebel et al. 2019). They estimated 10 origins of bird pollination in *Salvia*, with one corresponding to the crown of subg. *Calosphace*. This single shift to hummingbird pollination at the crown node of subg. *Calosphace* was followed by 56 reversals to bee pollination (and a few subsequent shifts back to hummingbird pollination). The results from these studies raise the possibility that bee-pollinated species derived from a bird-pollinated ancestor may have retained morphological similarities inherited from their hummingbird-pollinated ancestry. Such a finding would indicate that certain morphological traits, even if evolutionary constrained, do not limit subsequent pollinator switching in *Salvia*. The straight anther connectives and styles shared across most bee and bird-pollinated species in subg. *Calosphace* (Claßen-Bockhoff et al. 2004b; Claßen-Bockhoff 2017) are morphological traits that may have evolved in this fashion.

A major obstacle toward clarifying the relationship between floral morphology evolution and switches in pollination syndrome is quantifying the shape of the flower and its constituent parts and incorporating these data into a phylogenetic framework. For example, *Salvia* possess fused petals that form a showy flower that can vary from narrow, funnellform corolla tubes to strongly bilabiate and open corollas. *Salvia* exhibits variation in curvature of anther connectives and styles that together with corolla shape are thought to be the result of natural selection for specific pollen placement on pollinators (Fig. 1; Wester and Claßen-Bockhoff 2006a, 2007; Celep et al. 2020). Geometric morphometric approaches (Klingenberg 2010) offer the opportunity to quantify these continuously varying floral features. In the context of floral evolution, these approaches have been used to capture floral shape variation in, for example, the Lamiales (Alexandre et al. 2015; Joly et al. 2018), Brassicaceae (Gómez et al. 2016), Melastomataceae (Reginato and Michelangeli 2016), and Solanaceae (Smith and Kriebel 2018). A few morphometric studies have quantified floral shape or size in *Salvia* and tested hypotheses regarding the evolution of signal-reward correlations and floral optimization relative to different pollinators (Benítez-Vieyra et al. 2014, 2019; Strelin et al. 2017), but with limited sampling.

We use elliptic Fourier analysis (eFa) of floral shapes in *Salvia* for the first time to quantify corolla tube, anther connective, and style shapes across a total of 436 species, or ~44% of the genus. To dissect the evolutionary dynamics of floral disparity across *Salvia*, these morphometric data are then analyzed within a comparative phylogenetic framework that includes 519 species of *Salvia* (Kriebel et al. 2019). The overarching goal of this study is to investigate the role of pollinator type and geography in the

evolution of floral shape variation, disparity, and the existence of contingent evolution and evolutionary constraints in flower disparity. Four hypotheses are evaluated using a variety of PCMs.

1. Floral morphospace of NW *Salvia* is different than that of the OW species, and these differences are correlated to different pollinators in each region, especially the widespread occurrence of bird pollination in the NW;
2. Shifts in floral morphology accompany the numerous shifts between bee and bird pollination seen across *Salvia*;
3. Corollas, anther connectives (key constituents of the unique *Salvia* staminal lever), and styles evolved in correlated fashion from curved to linear shapes following shifts from bee to hummingbird pollination;
4. Evolutionary (developmental) constraint in floral features has occurred in *Salvia* subg. *Calosphace*, which would explain similarities (e.g., straight anther connectives and styles) between bee-pollinated species derived from bird-pollinated ancestry and their bird-pollinated relatives.

Materials and Methods

MORPHOMETRIC ANALYSES OF COROLLA, ANTHER, AND STYLE SHAPE IN *SALVIA*

The literature was searched for drawings of *Salvia* corollas (side views), anther connectives, and styles (e.g., Hedge 1974). This dataset was supplemented by scanning and photographing floral structures from species grown at the University of California Berkeley and Denver Botanical Gardens, the University of Wisconsin–Madison greenhouse, and field specimens (Fig. 1). A Canon LiDE 220 (LED Indirect exposure) flatbed scanner was used to scan flowers and their structures. Sampling was designed to encompass phylogenetic diversity across *Salvia*, targeting species from all major clades identified in previous phylogenetic studies (Walker et al. 2004; Walker and Sytsma 2007; Will and Claßen-Bockhoff 2014, 2017; Drew et al. 2017; Hu et al. 2018; Kriebel et al. 2019).

Side view shapes of corollas, anther connectives, and styles from *Salvia* and outgroups, *Lepechinia* and *Melissa*, were then converted into outlines, filled with black, placed on a white background, and saved in .jpg format using GIMP 2.8 (Solomon 2009; <http://www.gimp.org>). eFa of each structure was conducted with the package Momocs version 1.3.0 (Claude 2008; Bonhomme et al. 2014) for the R statistical environment (R Core Team 2018). This method of outline analysis has been used to quantify the shape of plant structures in a taxonomic and/or phylogenetic context with moss sporophytes (Rose et al. 2016), leaves (Kincaid and Schneider 1983; Chitwood and Otoni 2017; Stoughton et al. 2018), pollen grains (Bonhomme et al. 2013; Kriebel et al. 2017), corolla tubes (Smith and Kriebel 2018), and stamens and

styles (Reginato and Michelangeli 2016). The floral images were imported into R and the closed curve shapes from the outlines were described as periodic functions that transform and decompose each into a series of harmonics (Bonhomme et al. 2014; Caillon et al. 2018). Prior to the eFa, tests were made to determine if landmarks were necessary to constrain the position of the outlines to avoid alignment problems; if so, landmarked outlines were subjected to a Full Generalized Procrustes alignment in Momocs before the eFa. Finally, harmonic coefficients were calculated from the outlines and their variation summarized using Principal Components Analysis (PCA). The function `calibrate_harmonicpower_efourier` in Momocs was used to confirm that 99.9% of harmonic power was achieved with 32 harmonics.

SCORING POLLINATORS IN *SALVIA*

Species were scored for pollination syndrome based on the literature. The main source for pollinator scoring of NW taxa comes from a comprehensive list that includes the assignment of pollinator syndromes to 602 species of *Salvia* (~60% of the genus) (Wester and Claßen-Bockhoff 2011). Of these, 184 species were assigned to bird pollination, 351 to bee pollination, 67 to a polymorphic group mostly visited by both bees and birds or considered insufficiently known, and one, *Salvia whitehousei*, was thought to be possibly butterfly pollinated (Wester and Claßen-Bockhoff 2011). To avoid creating a category of butterfly pollination with a single species, and because its pollinator(s) has not been confirmed, we grouped *S. whitehousei* with the polymorphic group, where other unconfirmed species have been placed. *Salvia arborescens* from Hispaniola is the only species documented to flower strictly at night and is moth pollinated (Reith and Zona 2016). Because of a lack of morphological information for this species, it was not included in this study. Species considered as “intermediate” in the study of Wester and Claßen-Bockhoff (2011) are mostly both bee and bird pollinated, so they were scored as polymorphic; however, these represent a small percentage of the total number of species included in this study (corolla = 29/375 species; connective = 32/356 species; style = 19/275 species).

Additional studies were consulted to code insect pollination in NW and especially OW species. These included records of bee pollination and visitation by the families Andrenidae, Apidae, Colletidae, Halictidae, Megachilidae, and Melittidae (Visco and Capon 1970; Proctor and Yeo 1973; Read 1983; Hedström 1985; Dieringer et al. 1991; Ouborg and Van Treuren 1995; Corsi and Bottega 1999; Bottega and Corsi 2000; Ne’eman and Dafni 1999; Barrett et al. 2000; Ohashi 2002; Claßen-Bockhoff et al. 2003, 2004a; Herrera 2005; Reith et al. 2006, 2007; Shavit et al. 2009; Grace 2010; Wester and Claßen-Bockhoff 2011; Zhang et al. 2011; Cairampoma and Martel 2012; Espino-Espino et al. 2012, 2014; Celep et al. 2014, 2020; Özbek 2014; Zhang and Li

2014; Huang et al. 2015; Ott et al. 2016; Şenol et al. 2017; Ye et al. 2017; Giuliani et al. 2018). Species visited by flies are primarily bee pollinated (Celep et al. 2014, 2020) and were scored as such.

MACROEVOLUTIONARY FRAMEWORK

We tested all four hypotheses using PCMs. Uyeda et al. (2018) have argued that PCMs encompass two different approaches (“hypothesis-testing” and “data-driven”), that these methods vary in how effective they are in discovering plausible histories of singular or near-singular events, and that they should be used in complement rather than as alternatives. In this spirit, we used approaches that require a priori hypotheses regarding which species belong to which pollination syndrome and then testing for significant differences in their morphologies in a phylogenetic framework (“hypothesis-testing”; e.g., OUwie). We then used an approach in which the locations of regime changes are themselves estimated along with the parameters of an Ornstein-Uhlenbeck (OU) process (“data-driven”; e.g., I1ou). Thus, the four hypotheses were evaluated with a suite of approaches (morphometric data separated by categorical groups and OUwie modeling, detection of shift regimes using OU models, and Phylogenetic Generalized Least Squares Regression [PGLS]). Each of them may have limitations in discovering plausible histories but in concert should provide stronger evidence.

Species with available trait data for the three floral features were matched to the time-calibrated, maximum clade-credibility (MCC) phylogenetic tree of 528 species of *Salvia* and relatives (Kriebel et al. 2019), with nonmatching species drop-tipped as necessary. To account for uncertainty in the phylogenetic estimation, we also used 10 median trees from 10 distinct groves of trees (Kendall and Colijn 2016) identified from the postburnin posterior sample of Kriebel et al. (2019). The subsequent comparative analyses were averaged over these 11 trees to incorporate the uncertainty in phylogenetic parameters. For OUwie and PGLS analyses, the outgroup taxa were removed to ensure comparisons included only *Salvia* species.

FLORAL VARIATION IN *SALVIA* BY BIOGEOGRAPHIC AREA AND POLLINATOR

We first tested for differences between species in floral trait morphospace when categorized by geography and pollinator (Hypothesis 1) without explicit regard to phylogenetic relationships. *Salvia* species were assigned to area (NW or OW) and pollinator type (bee, bird, or polymorphic). The area of floral trait morphospace occupied by each group and their overlap was quantified by calculating the convex hull around each set of points using the `chull` function in R, then converting each convex hull into a polygon, and finally using the `st_area` and `st_intersection` functions of the R package `sf` version 0.7-3 (Pebesma 2018) to calcu-

late their area and overlap. To test for mean differences between the morphology of the species grouped by geography and pollinator, approximate randomization tests were performed using the `independence_test` function of the R library `coin` version 1.3-0 (Hothorn et al. 2008), resampling 10,000 times. For these tests, the scores of the first two PC axes of each of the three structures were used because the shape variation they describe is most pertinent to our hypotheses. The function `pairwisePermutationTest` of the R package `rcompanion` version 2.2.1 (Mangiafico 2019) was used to conduct multiple comparisons between the means of all three pollinator groups.

We then tested for differences in floral morphology between species of different areas and pollination syndromes by accounting for similarities in trait values due to common evolutionary history using PGLS (Grafen 1989; Symonds and Blomberg 2014) with the R library `nlme` version 3.1-139 (Pinheiro et al. 2019). The categorical predictor variables of geography and pollinator were tested against the first two continuous axes (PC1 and PC2) of morphometric data for each trait. Models in which the correlation structure followed a Brownian Motion (BM) (Felsenstein 1985; Martins and Hansen 1997), “lambda” (Pagel 1999), or an OU (Hansen 1997; Butler and King 2004) process were first compared using likelihood ratio tests with the `ape` library version 5.0 (Paradis and Schliep 2018). The variance explained by each model was assessed using the coefficient of determination (R^2) calculated with the R library `rr2` version 1.0.2 (Ives and Li 2018; Ives 2019). Phylogenetic sensitivity analyses were performed for PGLS models with the R packages `sensiPhy` version 0.8.4 (Paterno et al. 2018) and `phylolm` version 2.6 (Ho and Ané 2014). The function `tree_clade_phylm` was used to calculate the impact of influential clades on PGLS model estimates taking into account phylogenetic uncertainty across the 11 trees. Clades tested included those with more than five species and included the following subgenera: *Audibertia*, *Calosphace*, *Glutinaria*, “Heterosphace,” *Salvia*, and *Sclarea*.

FLORAL SHIFT REGIMES IN *SALVIA* RELATIVE TO CHANGES IN POLLINATOR

To test Hypothesis 2 that shifts in morphology match changes in pollinator, shifts in selective regimes for each floral trait were modeled under an OU process without requiring an a priori hypothesis of where changes in morphology may have occurred on the trees (“data-driven” sensu Uyeda et al. 2018). This analysis was done with the R library `I1ou` version 1.42 (Khabbazian et al. 2016), which uses the LASSO (Least Absolute Shrinkage and Selector Operator) (Tibshirani 1996) method to detect shifts in phenotypic optima on phylogenetic trees. The best shift configuration was selected using the phylogenetic Bayesian Information Criterion (pBIC), a conservative model when searching for evolutionary regime shifts (Khabbazian et al. 2016). For each

of the three floral traits (corolla, anther connective, and style), univariate I1ou analyses with PC1 scores and multivariate analyses with both PC1 and PC2 scores were done to examine shape dynamics across all 11 trees. To test for convergent evolution in I1ou, the detected shifts were collapsed to identify convergent regimes. To compare shifts in floral traits detected by I1ou with changes in pollination syndrome, we conducted ML ancestral state reconstruction (ASR) of pollinators with corHMM version 1.22 (Beaulieu et al. 2017). We performed ASR on the three sets of reduced trees matching species with available trait data. The All Rates Different model of discrete trait evolution was used in pollinator ASR because it was found to be the best fitting model with the same trees and data (Kriebel et al. 2019). The Yang rooting method was used to fix the root state probabilities with estimated transition rates (Yang et al. 1995; Yang 2006; Beaulieu et al. 2017).

FLORAL TRAIT CORRELATIONS IN *SALVIA*

To test Hypothesis 3 that corolla, anther connective, and style shapes evolved in a correlated fashion, we employed PGLS with the library nlme using PC1 and PC2 of each floral trait. Test of different evolutionary models for the correlation structure was carried out as described for previous PGLS analyses. We hypothesized that bird-pollinated *Salvia* would evolve straighter floral traits for easier nectar probing and accurate pollen placement. Likewise, bee-pollinated species would evolve curved anther connectives and styles for pollen placement on head or thorax. Because the common ancestor of the genus is bee pollinated (Kriebel et al. 2019), we expected the correlated changes to evolve from curved to straight shapes. Sensitivity analyses were performed using the `tree_clade_phylm` function in `sensiPhy` as described for previous PGLS analyses.

EVOLUTIONARY CONSTRAINT IN FLORAL TRAITS IN *SALVIA*

To test Hypothesis 4 that evolutionary constraint was operating to limit changes in floral shape despite pollinator changes within subg. *Calosphace*, we formed four groups of species: (1) bird pollinated, (2) bee pollinated but derived from bird-pollinated ancestry (mostly in subg. *Calosphace* and three species in NW “Heterosphace”), (3) bee pollinated, and (4) polymorphic. First, we used PGLS and sensitivity analyses with `sensiPhy` to test for differences in morphology between these four groups. Second, we examined six cases of continuous character evolution (one for each of six floral traits: PC1 and PC2 of corolla, anther connective, and style) informed by the four pollinator regimes using BM and OU models implemented in `OUwie` version 1.53 (Beaulieu et al. 2012). For each floral trait, we built seven different classes of models (two BM and five OU processes) in which up to three parameters were estimated: θ (the phenotypic optimum), α (the

strength of selection towards the optimum), and σ^2 (the evolutionary rate parameter). The seven models included a simple BM with constant σ^2 across the phylogeny (BM1), multiple-rate BM in which σ^2 can assume a different value for each regime mapped on the phylogeny (BMS), a simple OU model with a constant θ (OU1), and more complicated OU models in which the floral trait has different θ (OUM), θ and σ^2 (OUMV), θ and α (OUMA), and θ , α , and σ^2 (OUMVA) for each regime. Models were compared using the corrected Akaike information criterion weights (AICcw) that were summarized across the 11 trees. Because `OUwie` requires a priori mapping of pollinator on the phylogeny, we performed stochastic character mapping across all 11 trees with 528 species using the `make.simmap` function of the R library `phytools` version 0.7-18 (Revell 2012) and implementing the All Rates Different model of discrete character evolution. The function `drop.tip.simmap` was then used to match species with morphological data to each of the trees to produce pruned trees with identical mappings.

The presence of strong pollinator-mediated selection and the lack of evolutionary constraint on a given trait would be evident in two ways. First, significant differences in floral traits using PGLS and `OUwie` would be seen between bee-pollinated species derived from bird-pollinated ancestors and their bird-pollinated relatives. Second, no significant differences would be evident in floral traits among the two bee-pollinated groups. Conversely, evolutionary constraint on a floral trait in subg. *Calosphace*, despite pollinator switching, would be identified in two ways. First, no significant differences would be seen in floral traits between bee- and bird-pollinated species in subg. *Calosphace* (and the few in “Heterosphace”). Second, significant differences would be evident between the two bee-pollinated groups. Results from `OUwie` analyses can also shed light into similarities of the evolutionary optima of each pollinator group; that is, assessing if NW bee species derived from bird-pollinated ancestors have similar optima to their bird-pollinated relatives or to other bee-pollinated species.

Results

MORPHOLOGICAL VARIATION OF *SALVIA* COROLLA, ANTER CONNECTIVE, AND STYLE SHAPE

A total of 504 outlines representing 375 species from all major clades were obtained for eFa of corollas. The best landmark configuration to avoid twisting of the corollas included the placement of three landmarks on each corolla, one on the upper lip, one on the lower lip, and a third at the base. The PCA of the harmonic coefficients explained 72.8% of the variation in the first two PCs, with PC1 and PC2 accounting for 57.6% and 15.2%, respectively, of the variation. PC1 explains variation in morphology from corollas with narrow mouths that are shallowly bilobed to

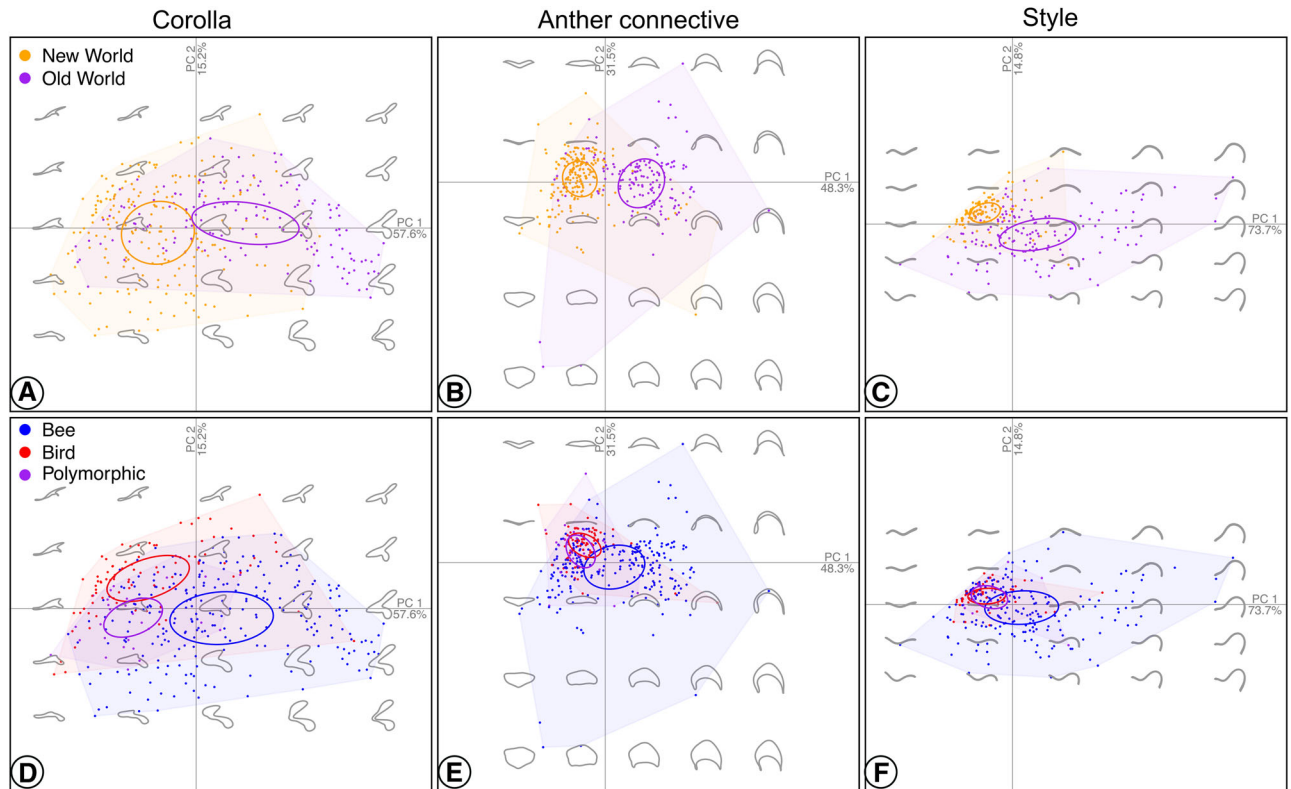


Figure 2. Morphospace variation of *Salvia* flowers from elliptic Fourier analyses of corolla tube (A and D), anther connective (B and E), and style (C and F). Specimens are grouped by geographic area (first row) and pollinator (second row). Shaded polygons represent total morphospace occupied and ellipses the 50% confidence ellipse for each area or pollinator. The sister clade comprising *Lepechinia* and *Melissa* is not shown for clarity but its position can be seen in Figure S1.

corollas that are broad and strongly bilobed (Figs. 2A and S1A). PC2 explains variation in corolla morphology from upper corolla lip slightly longer than the lower lip to corollas with a much longer lower lip. Both the landmarked and unlandmarked outlines of all floral traits are available in the data package accompanying this article (Dryad: <https://doi.org/10.5061/dryad.q573n5tg5>).

The eFa of anther connectives included 456 outlines from 356 species. The pollen-bearing anther was removed to achieve best results, as the anther attachment to the anterior arm of the connective created strong angles between both structures and produced noise during morphometric analyses. Four landmarks were placed to avoid twisting of the specimens during the eFa: two at the widest part of the connectives and two at the upper and lower parts in the middle section of the connective. The PCA of harmonic coefficients explained 79.8% of the variation in the first two PCs (PC1 = 48.3%; PC2 = 31.5%). PC1 explains variation in connectives from straight to strongly curved; PC2 explains variation from thin to thick anther connectives (Figs. 2B and S1B).

The eFa of styles included 319 outlines of 275 species. Prior to the analysis, stigmas were removed from all outlines just below the division of the lobes from the style. Four landmarks were

placed to avoid twisting of the styles during the eFa. The PCA of harmonic coefficients explained 88.5% of the variation in the first two PCs (PC1 = 73.7%; PC2 = 14.8%). PC1 explains variation from a straight to somewhat convex style to a curved style at the distal end, whereas PC2 explains variation toward the lower half of the style, from strongly curved to straight and concave (Figs. 2C and S1C).

FLORAL TRAIT VARIATION BY GEOGRAPHY

NW species generally exhibit narrower corollas with longer lower lips and shorter upper ones, straighter styles, and straighter and thicker anther connectives, whereas those from the OW occupy the area of morphospace characterized by strongly bilobed corollas and strongly curved styles and anther connectives (Fig. 2A-C). However, there is considerable overlap between the OW and NW flower morphology. Each geographical area occupies a similar size of corolla morphospace and share 58.6% of the total occupied area (Table S2.1). For anther connectives, OW and NW taxa are clustered on opposite sides of the morphospace (Fig. 2B). Specifically, strongly curved anther connectives are rare in NW taxa, and the straightest and thickest connectives are almost entirely restricted to NW species. OW

taxa also vary more, covering almost twice the area of morphospace relative to NW species (Table S2.1). Similar to the anther connectives, strongly curved styles are unique to OW species and the straightest styles are largely restricted to NW species where they are separately clustered in morphospace (Fig. 2C). OW taxa cover a much broader area of style morphospace than do NW taxa and both areas share 25.5% of the total occupied area (Table S2.1). Approximate randomization tests resulted in significant differences between the morphology of NW and OW species for both PC variables of all three traits except for the shape of the connective in PC2 (Table S2.1).

PGLS regressions, testing for differences between species of different geographical areas, were done on reduced trees of 257 species for corolla, 263 for anther connective, and 206 for style data. Likelihood ratio tests favored the implementation of the lambda model for the correlation structure in the PGLS regressions of all variables. Significant differences were found between species of the NW and the OW for PC1 of corolla ($P = 0.044$, $SD = 0.008$) and PC1 of style shape ($P = 0.019$, $SD = 0.006$) of the six variables tested (Table S2.3). Sensitivity analyses showed that the OW clades *Glutinaria*, *Salvia*, and *Sclarea* were influential on the parameters of some models (Table S2.2), but their exclusion did not change the results of significant differences in corolla PC1 and style PC1 (Dryad: <https://doi.org/10.5061/dryad.q573n5tg5>). The “Heterospace” clade that is the only one to include NW and OW species together in the same clade was found to be influential in both axes of connective shape (Table S2.2). The exclusion of “Heterospace” changed the result from no significant difference in connective shape between species of both geographic regions (connective PC1: $P = 0.673$, $SD = 0.171$; connective PC2: $P = 0.541$, $SD = 0.189$) to a significant difference between them when excluding this clade (connective PC1: $P = 0.06$, $SD = 0.021$; connective PC2: $P = 0.003$, $SD = 0.002$).

ASR: BEE POLLINATION ANCESTRAL WITH ONE MAJOR SHIFT TO BIRD POLLINATION

The All Rates Different model of discrete trait evolution coupled with the Yang rooting method resulted in a high probability (96–98%) of bee pollination as ancestral across the full 528 species trees and the three sets of reduced species trees matching trait data (Figs. 3–5). The main shift to bird pollination (hummingbirds) occurred with high probability (85–94%) on all trees at the crown of NW subg. *Calosphace* around 20 million years ago. This shift was subsequently followed by numerous reversals (22–35) back to bee pollination. The shifts to bird pollination inside NW subg. *Audibertia* and the OW/NW “Heterospace” clade are always reconstructed as being younger than 5 million years old (Ma).

FLORAL TRAIT VARIATION BY POLLINATOR

The overlap of bird- and bee-pollinated species in corolla morphospace is similar to the overlap between OW and NW species (Fig. 2D; Table S2.4). The area of morphospace occupied by bee-pollinated species is 13% larger than that occupied by hummingbird-pollinated species. However, the latter occupy a unique area of morphospace with very narrow and less bilobed corolla tubes, whereas bee-pollinated taxa have unique morphologies with strongly bilobed corollas or with relatively long lower corolla lobes (Fig. 2D; Table S2.4). Anther connectives of bee-pollinated species cover an area of morphospace almost five times that of bird-pollinated species, and the morphospace of bird-pollinated species is almost entirely embedded in the area occupied by bee-pollinated species (Fig. 2E; Table S2.4). However, most bird-pollinated species are clustered in a small area of anther morphospace that includes straight and thin anther connectives, whereas bee-pollinated species occupy larger morphospace. Although bee-pollinated species exhibit a wider morphospace, they are clustered in two opposite areas of morphospace—showing either straight or curved anther connectives. Styles of bee-pollinated species cover an area of morphospace almost six times that of bird-pollinated species (Fig. 2F; Table S2.4). Bird-pollinated species are clustered in the area of morphospace of straight and slightly curved styles, whereas the bee-pollinated species are spread throughout the morphospace. *Salvia* species occupying the region of morphospace with strongly curved styles are only pollinated by bees. Approximate randomization tests resulted in a significant difference for all traits ($P < 0.001$). Pairwise multiple comparisons gave significant differences for all three floral traits between bee- and bird-pollinated species (Table S2.4). Species scored as polymorphic in pollination were most similar to bird-pollinated species. These two pollinator groups were similar in the first axis of all three floral traits and polymorphic species exhibited significant differences from bee-pollinated taxa in all traits.

Likelihood ratio tests favored the lambda model for the correlation structure of the PGLS regressions between pollinator type and trait shape. Significant differences in floral morphology were found between pollination syndromes for PC1 of the corolla (bee vs. bird: $P < 0.001$, $SD < 0.001$; bee vs. polymorphic: $P < 0.001$, $SD < 0.001$) and for PC2 of the corolla (bee vs. bird: $P < 0.001$, $SD < 0.001$) (Table S2.5). Bee-pollinated species also differed from bird-pollinated taxa in PC2 of the anther connective ($P < 0.001$, $SD < 0.001$; Table S2.5). Sensitivity analyses indicated that the *Calosphace* clade was influential on model parameters of the PGLS regression comparing the shape of connectives as represented by PC2 and pollinators (Table S2.2). Removing *Calosphace* changes the result between bee and polymorphic species from a significant difference ($P <$

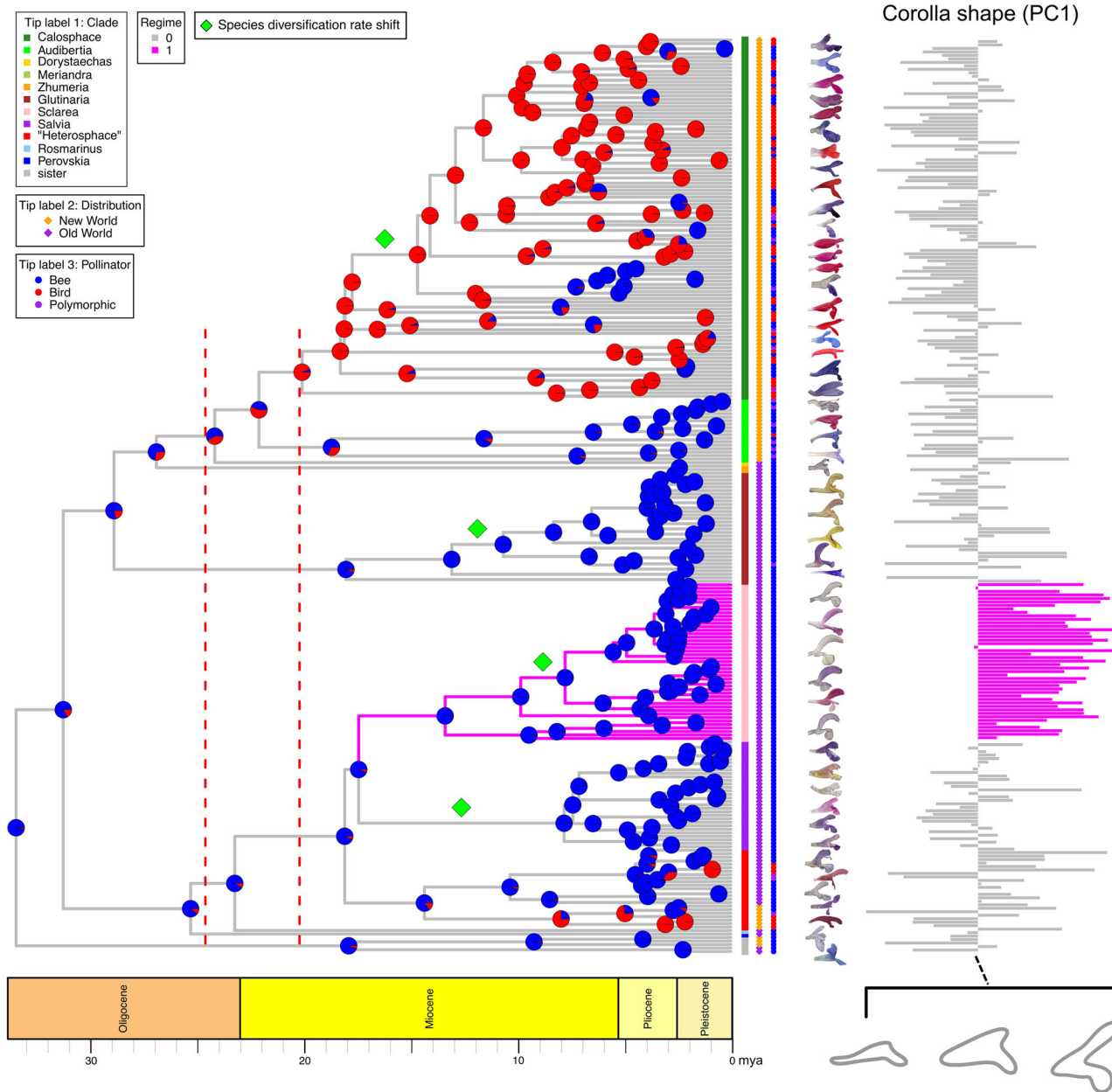


Figure 3. Univariate shift detection analysis with I1ou using PC1 from elliptic Fourier analysis of corolla tube in *Salvia* and close relatives. The left column depicts the phylogenetic tree with 262 tips based on the chronogram from Kriebel et al. (2019), with species dropped if not sampled. The four shifts in species diversification within *Salvia* from Kriebel et al. (2019) are indicated by green diamonds. Tip labels (left to right) provide subgeneric placement and scoring for geographic area and pollinator. The right column depicts the score of PC1 for each tip (as a bar from the mean score) for corolla tube shape. Below the barplot, a cartoon illustrates the mean (with its position indicated with a black dashed line) and the extreme shapes in PC1. Colored PC1 lines (and the correspondent clade edges on the phylogenetic trees) represent separate regimes from the best shift configuration under the pBIC model selection criterion. Specimen examples next to the tips of the tree are positioned approximately where the species falls in the phylogeny. Red dashed lines indicate the 95% confidence interval of the crown radiation of hummingbirds (McGuire et al. 2014).

0.001, SD < 0.001) to a nonsignificant one ($P = 0.308$, SD = 0.031). This may be due in part to the fact that most polymorphic taxa are in *Calosphace*. The strong significant difference between bee- and bird-pollinated taxa remained after removing *Calosphace*.

DO SHIFTS IN MORPHOLOGY OCCUR WHERE SHIFTS IN POLLINATION ARE INFERRED?

We asked whether significant shifts in floral trait evolution occurred with shifts in pollinators. The best shift configuration found by I1ou using PC1 of *Salvia* corolla tube shape

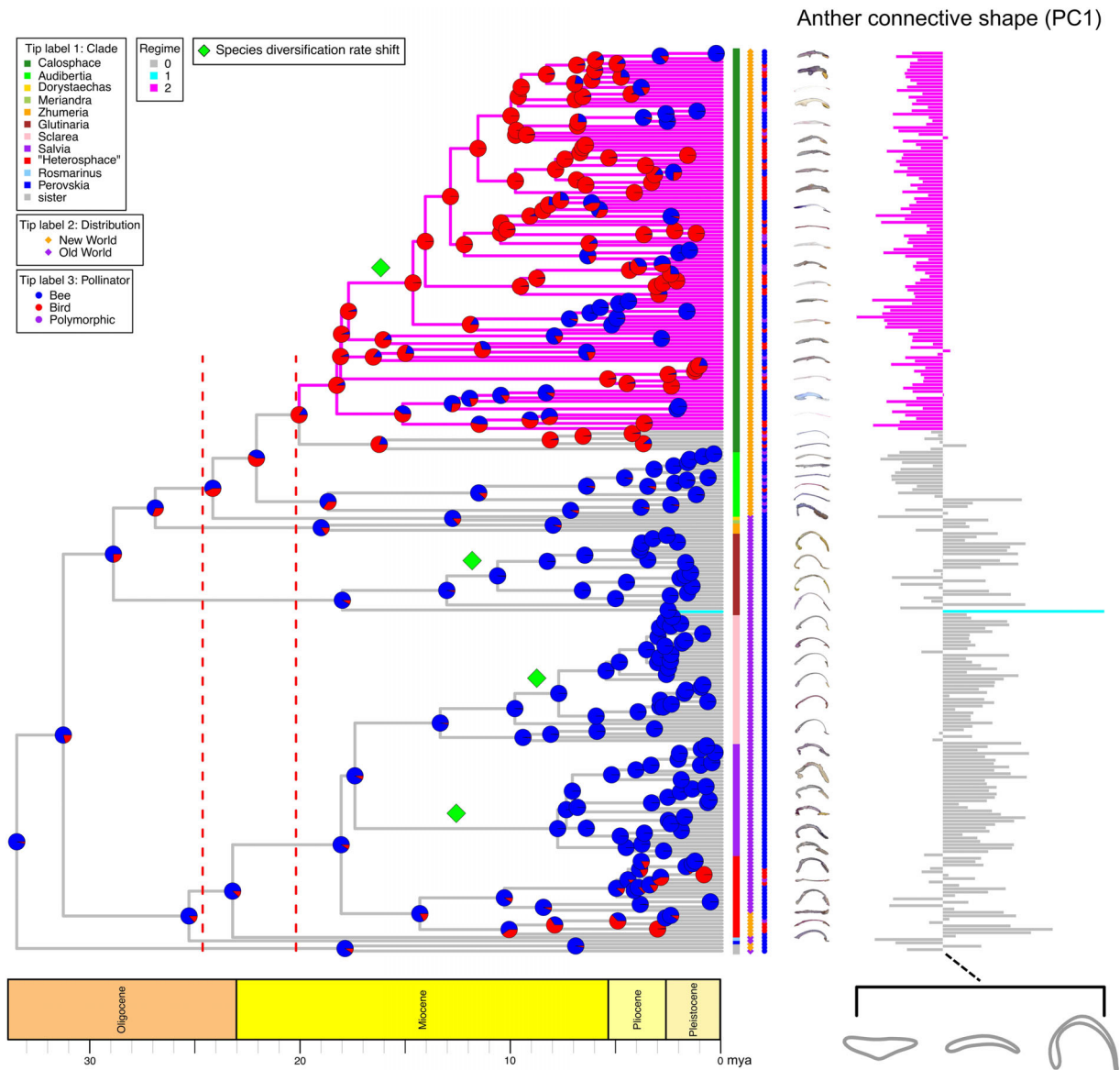


Figure 4. Univariate shift detection analysis with I1ou using PC1 from elliptic Fourier analysis of anther connective in *Salvia* and close relatives. The left column depicts the phylogenetic tree with 266 tips based on the chronogram from Kriebel et al. (2019), with species dropped if not sampled. The four shifts in species diversification within *Salvia* from Kriebel et al. (2019) are indicated by green diamonds. Tip labels (left to right) provide subgeneric placement and scoring for geographic area and pollinator. The right column depicts the score of PC1 for each tip (as a bar from the mean score) for anther connective shape. Below the barplot, a cartoon illustrates the mean (with its position indicated with a black dashed line) and the extreme shapes in PC1. Colored PC1 lines (and the correspondent clade edges on the phylogenetic trees) represent separate regimes from the best shift configuration under the pBIC model selection criterion. Specimen examples next to the tips of the tree are positioned approximately where the species falls in the phylogeny. Red dashed lines indicate the 95% confidence interval of the crown radiation of hummingbirds (McGuire et al. 2014).

included a mean of 1.7 (SD = 0.8) shifts per tree, with all trees including a shift to strongly bilobed and broad corolla tubes on the edge leading to OW subg. *Scleara*. The results on the MCC tree are shown in Figure 3 (results on the 10 grove trees provided in the data package accompanying this article). Subgenus *Scleara* and its sister clade are bee pollinated and thus this shift did not match a change in pollinator between bee and

bird. Two of the trees showed an additional shift to narrowly tubular and shallowly bilobed corollas on the edge leading to the large NW clade of subg. *Audibertia* + *Calosphace*. This shift precedes the switch to bird pollination inferred near the most recent common ancestor (MRCA) of subg. *Calosphace*. The second I1ou analysis with both PC1 and PC2 of corolla shape detected a mean of 5.5 (SD = 1.8) shifts per tree with

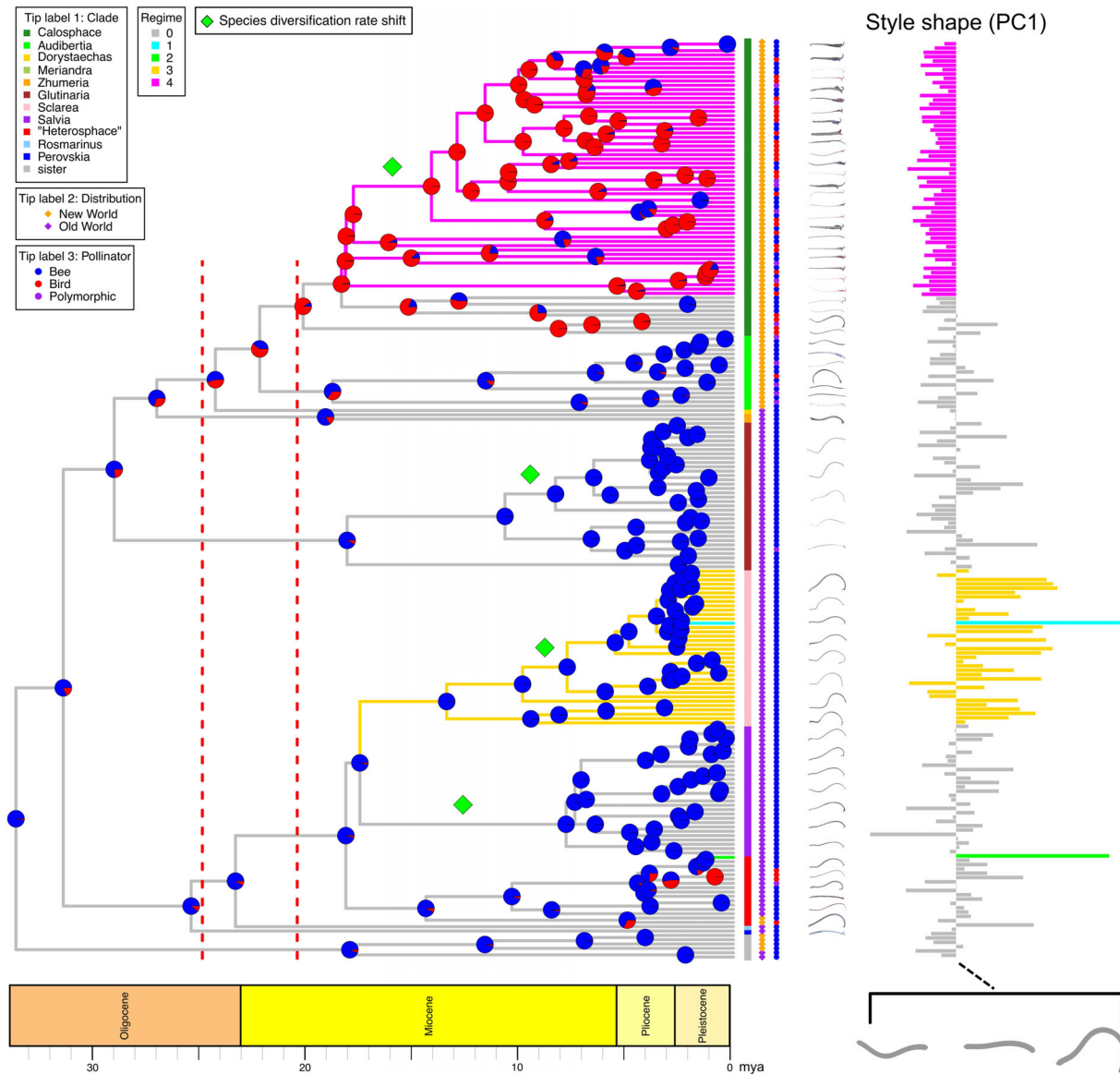


Figure 5. Univariate shift detection analysis with 11ou using PC1 from elliptic Fourier analysis of the style in *Salvia* and close relatives. The left column depicts the phylogenetic tree with 212 tips based on the chronogram from Kriebel et al. (2019), with species dropped if not sampled. The four shifts in species diversification within *Salvia* from Kriebel et al. (2019) are indicated by green diamonds. Tip labels (left to right) provide subgeneric placement and scoring for geographic area and pollinator. The right column depicts the score of PC1 for each tip (as a bar from the mean score) for style shape. Colored PC1 lines (and the correspondent clade edges on the phylogenetic trees) represent separate regimes from the best shift configuration under the pBIC model selection criterion. Specimen examples next to the tips of the tree are positioned approximately where the species falls in the phylogeny. Red dashed lines indicate the 95% confidence interval of the crown radiation of hummingbirds (McGuire et al. 2014).

two consistent shifts across the 11 trees (results on the MCC tree provided in Fig. S2). The first shift occurred on the edge leading to subg. *Sclarea* in 10 trees and the second shift occurred on the edge subtending a small clade of bee-pollinated species in NW subg. *Calosphace* in nine trees. This second shift is correlated to species with narrow tubes and long lower corolla lips and occurs simultaneously with a switch from bird to bee pollination.

The best shift configuration of anther connective shape with PC1 found a mean of 5.9 (SD = 1.9) shifts per tree. A shift to straight anther connectives is detected in all but three trees for one node within NW subg. *Calosphace* that defines the large majority of the subgenus (Fig. 4). This shift co-occurs with a switch from bee to bird pollination. In about half of the trees, a shift to straight anther connective shape is also detected within NW subg. *Audibertia* in about half of the trees and is convergent

with the one inside subg. *Calosphace*. A shift to curved anther connectives was found on most trees near the MRCA of a large OW clade that includes the subgenera “Heterosphace,” *Salvia*, and *Sclarea*. A convergent shift to these curved floral shapes is found within the East Asian subg. *Glutinaria*. The analysis with PC1 and PC2 found a mean of 14.6 (SD = 2.7) shifts per tree (results on the MCC tree provided in Fig. S3). A convergent shift to stout, reduced anther connectives is shared between the out-group genera (*Lepechinia* and *Melissa*) and several OW taxa previously recognized as distinct genera: *S. majdae* (*Zhumeria*), *S. yangii* (*Perovskia*), and *S. dorystaechas* (*Dorystaechas*). Anther connective shifts involving large clades include one on the edge leading to the MRCA of subgenera *Audibertia* + *Calosphace* and one within NW subg. *Calosphace* with straighter and thicker connectives. The latter shift occurs at the same node in which a shift in corolla shape (PC1 and PC2) is evident and co-occurs with a switch from bird to bee pollination.

The best 1100 shift configuration of style PC1 included a mean of 4.2 (SD = 0.4) shifts per tree. Three of these shifts were to strongly curved styles and all occurred in OW clades, particularly on the edge leading to subg. *Sclarea* (Fig. 5). The fourth shift to straight styles in all trees was placed one node after the switch to hummingbird pollination near the MRCA of NW subg. *Calosphace*. No convergent shifts were detected with PC1 of style shape. The analysis including PC1 and PC2 found a mean of 16.5 (SD = 2.7) shifts per tree. Most shifts across trees are in the predominately OW clade that includes subg. *Rosmarinus* + “Heterosphace” + *Salvia* + *Sclarea* and involve changes to more curved styles (results on the MCC tree provided in Fig. S4). Further shifts in style curvature were observed in subg. *Sclarea*. A shift to straighter styles near the MRCA of subg. *Audibertia* + *Calosphace* is seen in eight trees (see results in the data package accompanying this article). Most style shape shifts do not co-occur with switches in pollinator. However, the style shape shift near the MRCA of *Audibertia* + *Calosphace* does precede the switch to hummingbird bird pollination in subg. *Calosphace*. Convergent shifts with PC1 and PC2 involved few and isolated species across OW subg. *Sclarea*, East Asian subg. *Glutinaria*, and NW subg. *Calosphace* and showed convergences in curvature to different regions of the style.

DO FLORAL TRAITS EVOLVE IN CORRELATED FASHION?

Third, we used PGLS regressions to test whether corollas, anther connectives, and styles exhibit significant correlation in trait evolution, perhaps in response to switches in pollinator. Pairwise comparisons involved 216 species for corolla and anther connective shape, 195 species for corolla and style shape, and 187 species for anther connective and style shape. Likelihood ratio tests favored the implementation of the lambda model for the cor-

relation structure in the PGLS regressions. Correlated evolution involving trait changes from curved to straight shapes in corolla, anther connective, and style was detected in each of the three tests using PC1 (corolla vs. anther connective: $P = 0.018$, SD = 0.006; corolla vs. style: $P < 0.001$, SD < 0.001; anther connective vs. style: $P = 0.016$, SD = 0.003; Table S2.6). Correlated evolution was also found between PC2 of the corolla and PC2 of the anther connective ($P < 0.001$, SD < 0.001) and indicates that corollas that have straighter tubes and galeas with longer lower corolla lobes evolve in concert with thicker anther connectives. Corolla PC2 also showed correlated evolution with style PC1 ($P = 0.015$, SD = 0.001) and PC2 ($P = 0.025$, SD = 0.004). Thus, decrease in curvature of the style is correlated to the change in corolla from short tubes with short upper lips and long lower lips to long narrow tubes with equally long upper and lower lobes. Changes from curved to straight anther connectives (PC1) were correlated with a secondary curvature toward the base of the style (PC2) ($P = 0.042$, SD = 0.01). Finally, changes from thin to thick connectives (PC2) were found to evolve in a correlated fashion with the overall shape of the style (PC1) ($P = 0.042$, SD = 0.009). Most of the pairwise comparisons that did not show correlated evolution involved the corolla (Table S2.6). Sensitivity analyses detected influential clades (Table S2.2) but their removal did not change the overall results. Corolla shape evolution thus appears to be more decoupled than does anther connective and style shape evolution.

IS THERE EVIDENCE FOR EVOLUTIONARY CONSTRAINT IN THE FLORAL TRAITS OF NW SALVIA subg. CALOSPSPACE?

Fourth, we asked whether bee-pollinated species in NW subg. *Calosphace* (and 1-3 NW species of “Heterosphace”) derived from hummingbird-pollinated ancestors exhibit similarity in their floral traits to other NW and OW bee-pollinated species or to hummingbird-pollinated species. Strong morphological differences in all three floral traits between bee-pollinated species with and without a bird-pollinated ancestry (Fig. 6) support the hypothesis that the legacy of a bird-pollinated ancestor played a role in shaping the novel floral morphology evident in bee-pollinated species of NW subg. *Calosphace*. The corolla lobing of bee-pollinated species arising out of bird-pollinated ancestry is more similar to that of bird-pollinated species and quite different from the strongly bilobed corollas of other NW and OW bee-pollinated species (Fig. 6A, PC1 axis). Similarly, the straight anther connectives of bee-pollinated species from subg. *Calosphace* are only shared with NW bird-pollinated species and differ from all other NW and OW bee-pollinated species (Fig. 6B, PC1 axis). However, they differ from the bird (and other bee)-pollinated species in having thicker connectives (Fig. 6B, PC2 axis). Finally, the straight styles of all subg. *Calosphace* species (bird, bee, or

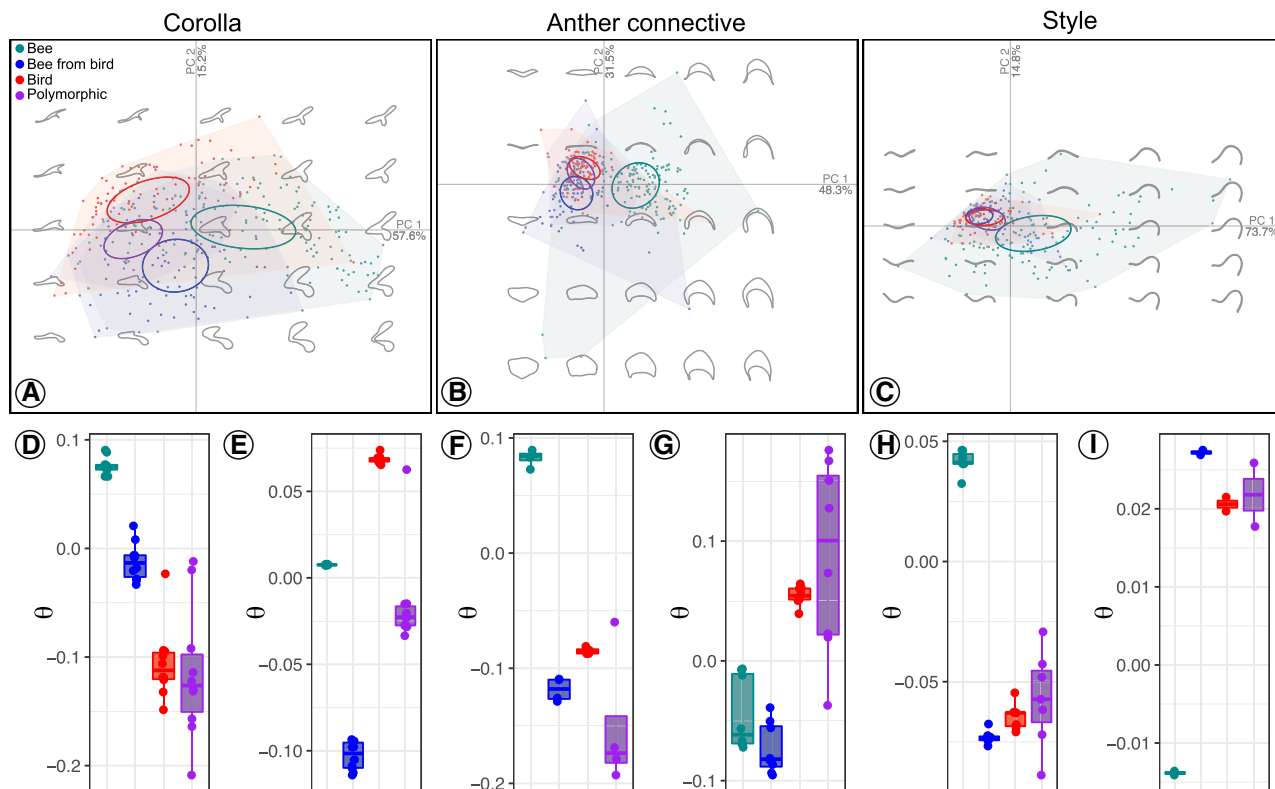


Figure 6. Morphospace variation of *Salvia* flowers from elliptic Fourier analyses of corolla tube (A), anther connective (B), and style (C). Species are placed into four groups of pollinators: bee pollinated but derived from a bird pollinated ancestor (only in subg. *Calosphace*), other NW and OW bee pollinated, bird pollinated, and polymorphic. Shaded polygons represent total morphospace occupied and ellipses the 50% confidence ellipse for each pollinator group. The sister clade is not shown for clarity but its position can be seen in Figure S1. Panels D-I depict boxplots of results from OUwie analyses comparing evolutionary optima (θ) by pollinator group for PC1 and PC2 of corolla (D-E), PC1 and PC2 of anther connective (F-G), and PC1 and PC2 of style (H-I).

polymorphic pollinated) are indistinguishable in morphospace, whereas other NW and OW bee-pollinated species have curved styles (Fig. 6C). Thus, anther connective shape, style shape, and corolla lobing (to a lesser degree) strongly support the hypothesis that bee-pollinated species derived from a bird-pollinated ancestor in subg. *Calosphace* have retained key features of the NW bird flower despite subsequent switches to bee pollination.

OUwie analyses provided additional strong support for the retention of these floral traits in bee-pollinated species derived from bird-pollinated ancestry. OUwie analyses were used to fit and compare seven evolutionary models and estimate model parameters for the four groups (bird, bee derived from bird, bee, and polymorphic), with OU models preferred over BM models for all six traits. Specifics on the OU models selected for each trait, their AICcw scores, adaptive optimum θ , variance σ^2 , and adaptation rate α values, and stochastic mapping of each trait over all trees are available elsewhere (Figs. S5 and S6; Dryad: <https://doi.org/10.5061/dryad.q573n5tg5>). Some of the more complex OU models did not converge and had negative eigenval-

ues in the Hessian matrix so they were discarded. The most complex model OUMVA was the best fit for two traits (corolla PC1: median AICcw = 0.992 across trees; anther connective PC1: median AICcw = 1 across trees; Table S2.8; Fig. S6). The OUMV model was the best fit for three traits (anther connective PC2: median AICcw = 1 across trees; style PC1: median AICcw = 1 across trees; style PC2: median AICcw = 1 across trees) and the OUM model was selected for the last trait (corolla PC2: median AICcw = 0.851 across trees).

The evolutionary optima for corolla lobing (PC1, Fig. 6D) of bee derived from bird and bird-pollinated species were similar and most different for all other bee-pollinated species. Similarly, the evolutionary optima for anther connective PC1 (Fig. 6F) for NW subg. *Calosphace* bee- and bird-pollinated species (sharing straight connectives) were similar and quite different from other bee-pollinated species. However, as seen with the morphospace analysis, all bee-pollinated species have similar evolutionary optima for anther connective PC2 that involves connective thickness (Fig. 6G). Finally, the evolutionary optima for style shape in both

PC1 (Fig. 6H) and PC2 (Fig. 6I) exhibit striking similarity between bee-pollinated species derived from bird ancestry and their bird-pollinated relatives, with the values of other bee-pollinated species quite different.

PGLS regression analyses using these four pollinator groups were less discriminating overall relative to the OUwie analyses (Table S2.7). This may be due in part to two clades that contain the majority of hummingbird-pollinated species, subg. *Calosphace* and “Heterosphace.” These two clades were significantly influential in the regression models for most traits in the sensitivity analyses (Table S2.2). Bee-pollinated species derived from bird-pollinated ancestry showed significant differences relative to other NW and OW bee-pollinated species in corolla shape in PC2 ($P < 0.001$, $SD < 0.001$) and style shape in PC1 ($P = 0.034$, $SD = 0.008$). Bee-pollinated taxa derived from a bird-pollinated ancestor showed significant differences from their bird-pollinated relatives in overall corolla shape (PC1: $P = 0.001$, $SD = 0.001$; PC2: $P < 0.001$, $SD < 0.001$) and as expected in anther connective shape PC2 ($P < 0.001$, $SD < 0.001$). The removal of the *Calosphace* and “Heterosphace” clades had an effect on the results of models including connective shape. When *Calosphace* is removed, bee derived from bird species show a significant difference to the other groups, but the removal of *Calosphace* involves eliminating the bee derived from bird and bird groups almost in their entirety. Removing “Heterosphace” on the other hand results in a change from nonsignificant ($P = 0.185$, $SD = 0.054$) to significant ($P = 0.051$, $SD = 0.014$) in the comparison of bee derived from bird and bee-pollinated taxa. This change in results highlights differences in the morphology of bee derived from bird taxa in *Calosphace* and “Heterosphace,” where the former has straight connectives and the latter more curved ones. Thus, on the basis of these three separate tests, the evolution of style and anther connective shape appears particularly constrained in bee-pollinated species derived from a bird-pollinated ancestor in subg. *Calosphace*. The evolution of straight styles and anther connectives near the base of subg. *Calosphace*, both adaptations to hummingbird pollination, did not inhibit subsequent pollinator switching in *Salvia*.

Discussion

CORRELATED EVOLUTION OF FLORAL TRAITS TO DIFFERENT POLLINATORS

The high diversity of angiosperms is frequently attributed to selection on their flowers by animal pollinators (Stebbins 1970; Crepet 1984; Barrett et al. 1996; Sargent 2004; van der Niet and Johnson 2012; Armbruster 2014; O’Meara et al. 2016). Selection, however, operates on an integrated set of floral traits involving the perianth and both male and female structures. Flowers thus often display a suite or “syndrome” of traits (e.g., shape, size, color,

scent, and timing) that evolve conjointly for effective pollination by “functional” guilds of pollinators (Fenster et al. 2004; Johnson 2010). A number of macroevolutionary scale studies have demonstrated that the evolution of corolla form is correlated to changes in pollinating vector (Whittall and Hodges 2007; Tripp and Manos 2008; Wessinger et al. 2014; Lagomarsino et al. 2017; Serrano-Serrano et al. 2017; Strelin et al. 2017; Joly et al. 2018; Smith and Kriebel 2018). We extend this finding by demonstrating that a larger suite of floral traits involving corolla, anther connective, and style shape is correlated in part with bee and bird pollinators in *Salvia*. However, the correlative evolution of these three traits and to pollinator in *Salvia* is more complex for two reasons. First, different parts of a trait (e.g., PC1 vs. PC2 of anther connective) can show different results in PGLS analyses. Second, evolutionary constraint can weaken the correlation signal in some traits (e.g., anther connective and style shape in subg. *Calosphace*) as discussed below.

We consistently found some overlap in trait space of bee- and bird-pollinated species, distinct areas of morphospace occupied by each syndrome, and significant differences between them. For all traits, there is a broader area of morphospace occupied by bee-pollinated species than by bird-pollinated counterparts (13% more for corolla, 69% more for connective, and 70% more for style). This pattern is particularly noticeable in the anther connective (Figs. 4E and 4F) and style (Figs. 4H and 4I), and suggests stabilizing selection by hummingbird pollinators. Corolla shape in particular was found to differ in shape between pollination syndromes even after accounting for common ancestry. Pollinators apparently have been instrumental in selection for changes in corolla and both male (anther connective) and female (style) function in *Salvia*. As ovule number is only four in *Salvia* (as in most Lamiaceae) and reproductive output is probably assured with a single pollinator visit, it is likely that changes in corolla form may be under more intense male rather than female selection via pollination (see also Aximoff and Freitas 2010; Minnaar et al. 2019). Indeed, Cruden (1977) noted that *Hedeoma* (also Lamiaceae) showed intraspecific variation in its pollen-ovule ratio (with only pollen number varying) and that this ratio directly correlated to corolla size.

We also examined using I1ou whether significant shifts in corolla, anther connective, and style shape mirror shifts in pollinators. One example involves the switch to hummingbird pollination and to straighter anther connectives and styles near the crown of subg. *Calosphace*. An additional shift within this subgenus from bird to bee pollination was accompanied by both changes in corolla and anther connective shape. However, many significant shifts in floral morphology occurred without switches in broad categories of pollinator. For example, we found strong support for the hypothesis (Himmelbauer and Stibal 1932, 1933, 1934) that *Salvia* species with strongly bilabiate corollas and a curved

dorsal lip or galea are derived from ancestors with an almost flat dorsal lip. An evolutionary shift to the extreme of this “Sclarea” type corolla is seen at the crown of subg. *Sclarea* but it is not correlated to a shift in pollinator as it occurs in the background of ancestral bee pollination (Fig. 3). Species in this subgenus have curved anther connectives hidden in the curved galea of the flower. This is a design that functions as a long lever that places pollen in a more extreme nototribic (dorsal or upper) position on bees and may thus reduce competition for pollinators as the result of differential pollen placement resulting in mechanical isolation (Celep et al. 2020). For this reason, these strongly bilobed flowers have been suggested to be the “ultimate response” to bee pollination (Westerkamp and Claßen-Bockhoff 2007). The link of this specialized floral trait combining corolla, anther connective, and style shapes to increased speciation is plausible as suggested by Westerkamp and Claßen-Bockhoff (2007). The greatest shift in speciation rate within *Salvia* occurs just after this shift in floral morphology in subg. *Sclarea* (Fig. 3; Kriebel et al. 2019). Future studies applying SSE models could help test the hypothesis that morphological changes detected here may be associated with increases in speciation rates.

DO SWITCHES IN POLLINATOR IMPOSE LONG-TERM EVOLUTIONARY CONSTRAINTS IN FLORAL MORPHOLOGICAL DISPARIFICATION?

Previous studies in *Salvia* have provided evidence for considerable evolution in corolla, anther connective, and staminal lever shape across the genus (Himmelbauer and Stibal 1932, 1933, 1934; Claßen-Bockhoff et al. 2004b; Walker and Sytsma 2007). One of the patterns uncovered by these studies was that straight staminal levers are present in both bee- and bird-pollinated species of NW subg. *Calosphace* (Claßen-Bockhoff et al. 2004b) despite numerous shifts back to bee pollination within the clade (Kriebel et al. 2019). We thus hypothesized that similarities observed in bee and bird-pollinated species in subg. *Calosphace* were due to constraints imposed by the early evolution (20 million years ago) of bird pollination in this clade. Support for this hypothesis is evident in floral morphospace analyses when bee-pollinated species in subg. *Calosphace* derived from hummingbird ancestry are separated from all other bee-pollinated species (Fig. 6A-C). The lack of strongly bilobed corollas and the straighter anther connectives and styles of these bee-pollinated species are more similar to the traits possessed by hummingbird species than those by other bee-pollinated species. OUwie models show that the evolutionary optima of all three floral traits are more similar between bird- and bee-pollinated species from subg. *Calosphace* and are quite different between the two bee-pollinated groups (Fig. 6D-I). The retention of these floral trait features following the switch to hummingbird pollination early in the evolution of subg. *Calosphace* clearly did not limit subse-

quent pollinator switching. It is noteworthy that one floral trait does track the switches to bee pollination. All bee-pollinated species in *Salvia* have similar evolutionary optima for thick anther connectives (PC2 axis). Thus, the staminal lever appears to have a trait that is evolutionary constrained (straightness of anther connective) and a trait that is not (thickness of anther connective).

Stebbins (1974) articulated the idea of evolutionary constraint in a complex structure comprising a suite of integrated characters when he stated that “*evolutionary canalization can be defined as the tendency for populations to respond adaptively to new environments in ways that are determined by characteristics acquired as a result of a previous adaptive radiation.*” Futuyma (2010) interpreted the idea of “constraint” more as “restraint” in possible directions of adaptive evolution. Joly et al. (2018) provided evidence for long-term evolutionary constraint on corolla shape in hummingbird- and bat-pollinated clades in Caribbean Gesneriaceae (Joly et al. 2018). In NW *Salvia* subg. *Calosphace*, however, the evolutionary constraint in floral features that arose at the time of a switch to hummingbird pollination 20 million years ago appears to be maintained despite numerous subsequent switches to bee pollination. This large clade evolved morphologies of three floral features (weakly bilabiate corolla, straight anther connective and style) that are likely adaptive for hummingbird pollination. These striking changes in floral morphology are seen nowhere else in *Salvia* and are largely retained even after subsequent switches back to bee pollination within subg. *Calosphace*. Changes in floral traits in response to bee pollination in species derived from bird-pollinated ancestry appear “constrained” and do not exhibit the morphology of OW and other NW species with strongly bilobed corollas and more curved anther connective and style traits.

Three additional traits present in both bee- and bird-pollinated species of subg. *Calosphace* have been suggested to unite this clade (with a few exceptions) and thus may have evolved along with the straightening of the connectives and styles. The first is the fusion of the anther connective arms for most of their length (Walker and Sytsma 2007). This character has been noted as a possible adaptation for hummingbird pollination in which “the posterior lever arms act as an abutment against the bird’s bill, a function which is optimized by their fusion” (Wester and Claßen-Bockhoff 2007). The continued presence of fused connective arms in bee-pollinated species in subg. *Calosphace* (see Fig. 1A) may be an additional constraint that has led to markedly different floral morphologies of NW and OW bee-pollinated taxa. The second and third floral traits shared by most species in this NW clade regardless of pollinator are the presence of a longer upper stigma lobe and a stigmatic brush (El-Gazzar et al. 1968). These two traits may also be constrained from a hummingbird-pollinated

ancestor, but their distributions and functions in *Salvia* have yet to be studied.

Why is such strong evolutionary constraint operating in floral traits of *Salvia* subg. *Calosphace*? There are several hypotheses that might explain these differences. The first is that bee-pollinated species derived from a bird-pollinated ancestor have not reverted back to OW bee-pollinated floral forms because they can continue to rely on secondary hummingbird pollination with their relatively straight flowers. Although plausible, we suggest that this cannot explain fully the unique suite of floral traits seen in NW subg. *Calosphace*. Although a few species of subg. *Calosphace* are demonstrated to be pollinated by both hummingbirds and bees (and scored as such in these analyses), a wealth of field observations across many subclades of the clade clearly identifies the effective, single pollinator type of each species (McGregor 1899; Wester and Claßen-Bockhoff 2006a, 2007, 2011; Dieringer et al. 1991; Benitez-Vieyra et al. 2019).

A second hypothesis is that the bee faunas of the OW and NW are functionally different to a degree that they exert different selective pressures on the floral morphology of bee-pollinated OW *Salvia* versus NW subg. *Calosphace*. We have found no evidence to suggest the bee faunas are strikingly functionally different between the NW and OW; indeed, the bee families Andrenidae, Apidae, Colletidae, Halictidae, and Megachilidae involved in *Salvia* pollination are common across the NW and OW (Danforth et al. 2013; Hedtke et al. 2013; Sydenham et al. 2017). The only family of bees missing in the NW is the small-sized Melittidae, and there are only a few observations of these visiting OW *Salvia* (Özbek 2014). On the other hand, documented bee pollination observations in both the OW and NW (including bee-pollinated species derived from hummingbird-pollinated ancestors) indicate that the most common bee pollinators of *Salvia* in both the NW and OW are the bee genera *Anthidium*, *Anthophora*, *Bombus*, *Megachile*, *Osmia*, and *Xylocopa* (Grant and Grant 1964; Visco and Capon 1970; Hedström 1985; Dieringer et al. 1991; Corsi and Bottega 1999; Claßen-Bockhoff et al. 2004a; Shavit et al. 2009; Zhang et al. 2011; Celep et al. 2014, 2020). The OW *Apis mellifera*, which was introduced into the NW, is another commonly recorded visitor to *Salvia* in both the NW and the OW.

The third hypothesis is that the strong morphological differences between NW bee-pollinated species derived from bird ancestry and all other NW and OW bee *Salvia* species are indeed the legacy of constraints imposed by the shift 20 million years ago to hummingbird pollination in the NW that defines subg. *Calosphace*. Stebbins' (1970) remarkable insight on the evolution of the flower and its pollinators is cogent here: "... they do not retrace the original evolutionary pathway with respect to the details of the structure, but only with respect to general adaptation." We demonstrate that a suite of floral features in NW

bee-pollinated species derived from an earlier switch from bee to hummingbird pollination is morphologically distinct from the floral features of other NW and OW *Salvia* clades that are ancestrally bee pollinated. A similar conclusion was reached in a combined morphological and comparative phylogenetic study of NW *Ruellia* (Acanthaceae), which exhibits a similar biogeographical distribution to *Salvia* subg. *Calosphace* (Tripp and Manos 2008). They provided compelling evidence that flowers of *Ruellia* have evolved multiple means of achieving the end product of bee pollination. They demonstrated that transitions to bee pollination within hummingbird-pollinated clades were accompanied by "historical baggage" in the retention of ancestral and hummingbird specific floral traits.

CONTINGENT EVOLUTION IN FLORAL MORPHOLOGICAL DISPARITY

A remarkable feature of the evolution of the flower in angiosperms is the apparent contingent nature of the emergence of new traits in the background of earlier traits and their subsequent diversification (Donoghue et al. 1998; Donoghue 2005; Armbruster and Muchhala 2009; Givnish et al. 2014). O'Meara et al. (2016) have shown that three contingent traits, corolla presence, bilateral floral symmetry, and reduced stamen number, function together as the most significant key innovation correlated with species diversification in angiosperms. These three features simultaneously constrain the approach of pollinators, modulate placement of pollen, and promote correspondence between pollen placement and stigmatic contact (Darwin 1862; Neal et al. 1998; Walker-Larsen and Harder 2000; Armbruster et al. 2009), and thus increase opportunities for prezygotic isolation and speciation (Donoghue et al. 1998; Armbruster and Muchhala 2009). *Salvia* exemplifies a species-rich clade possessing these three key contingent features but then evolving a subsequent innovation, the staminal lever. The elongated anther connective examined here is the critical feature of the staminal lever that is seen in most (but not all) *Salvia*. The staminal lever arose via a nested series of contingent events. First, a correlated shift from radial to bilateral symmetrical flowers and the reduction from five to four stamens occurred in Lamiales and Lamiaceae (Refulio-Rodriguez and Olmstead 2014; O'Meara et al. 2016). Second, stamen number was reduced from four to two at the crown of *Salvia* (Drew et al. 2017) and in a parallel fashion multiple times within the subtribe Menthineae (Drew and Sytsma 2012). Third, the anther connective elongated once (with reversals) or several times in *Salvia* (Walker and Sytsma 2007). Notably, these three contingent steps appear to have arisen in parallel within the "justicoid" clade in the Acanthaceae of the order Lamiales (Kiel et al. 2017).

The evolution of the staminal lever system in *Salvia* is complex and this shape analysis of the anther connective only begins

to unravel the evolutionary history of this remarkable and almost singular structure in flowering plants. As a complex structure, assessing homology in the staminal lever can be difficult (Claßen-Bockhoff et al. 2003, 2004a,b; Walker and Sytsma 2007; Huang et al. 2015). The results presented here, however, support earlier suggestions (Himmelbaur and Stibal 1932, 1933, 1934; Claßen-Bockhoff et al. 2004a) that the anther connective elongated and was variously modified in different clades. This elongation was radically reduced in the formerly recognized genera *Dorystaechas*, *Meriandra*, *Perovskia*, and *Zhumeria*, all now considered subgenera within *Salvia* (Walker and Sytsma 2007; Drew et al. 2017; Kriebel et al. 2019). Our results also demonstrate parallel evolution of the distinctive lever with strongly curved anther connectives. This lever type had previously defined subgenus *Sclarea* but is now understood to be present in three subgenera.

Is the staminal lever in *Salvia* a “key innovation” (Claßen-Bockhoff et al. 2004a) that led to an “adaptive radiation” (sensu Givnish 2015)? An emerging view of the evolution of traits and their successes argues that it is more profitable to shift away from the view of “key innovations” as singular events and toward the view that they are complex features contingent on other traits and the specific environmental contexts in which they arise (e.g., de Queiroz 2002; Donoghue 2005; O’Meara et al. 2016). The staminal lever in *Salvia* involves a complex set of features as we have demonstrated here only examining the anther connective portion of the structure. The staminal lever has not evolved in isolation but rather in concert with both the corolla and style, under different pollinator pressures and evolutionary constraints, and in a striking contingent fashion.

AUTHOR CONTRIBUTIONS

RK, BD, and KJS conceived of the project. BD and KJS provided the funding for the project. RK, BD, JGG-G, FC, LH, and MMM obtained the morphometric data. RK, BD, and KJS provided the calibrated phylogenetic tree. RK performed the analyses. RK and KJS wrote the first draft of the manuscript, and BD edited the draft. All authors edited the final version.

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DATA ARCHIVING

Species, vouchers, and GenBank accessions used for the phylogenetic tree are deposited in Dryad (<https://doi.org/10.5061/dryad.8m40rb0>). The R scripts, morphometric data for corolla, anther connective, and style, and pollinator scoring are deposited in Dryad (<https://doi.org/10.5061/dryad.q573n5tg5>).

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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. Morphospace from Figure 2D–2F of corolla (A), anther connective (B), and style (C) showing the position of the sister group to *Salvia*, the genera *Lepechinia* and *Melissa*.

Tab. S2.1. Morphospace occupation of *Salvia* floral traits by geographical area and by pollinator.

Tab. S2.2. Results of sensitivity analyses testing for the influence of the major clades of *Salvia* on model parameters of Phylogenetic Generalized Least Squares regression models.

Tab. S2.3. Phylogenetic Generalized Least Squares (PGLS) regression models comparing the morphology of three *Salvia* floral traits represented by the first two PCs each by geographical distribution.

Tab. S2.4. Morphospace occupation of *Salvia* floral traits by pollinator.

Tab. S2.5. Phylogenetic Generalized Least Squares (PGLS) regression models comparing the morphology of three *Salvia* floral traits represented by the first two PCs each by pollinator.

Tab. S2.6. Phylogenetic Generalized Least Squares (PGLS) regression models testing for correlated evolution between the main axes (PC1 and PC2) of corolla, anther connective and style morphology.

Tab. S2.7. Phylogenetic Generalized Least Squares (PGLS) regression models comparing the morphology of three *Salvia* floral traits represented by the first two PCs each by pollinator with bee pollinated species derived from a bird pollinated ancestor grouped separately.

Tab. S2.8. Median AICc weights (AICcw) representing the relative likelihood of each of the seven evolutionary models fitted to PC1 and PC2 of corolla, connective and style shape by pollinator.

Fig. S2. Multivariate shift detection analysis with I1ou using PC1 and PC2 from elliptic Fourier analysis of corolla tube shape in *Salvia* and close relatives.

Fig. S3. Multivariate shift detection analysis with I1ou using PC1 and PC2 from elliptic Fourier analysis of anther connective shape in *Salvia* and close relatives.

Fig. S4. Multivariate shift detection analysis with I1ou using PC1 and PC2 from elliptic Fourier analysis of style shape in *Salvia* and close relatives.

Fig. S5. Results from stochastic mapping of pollinator under the All Rates Different (ARD) model of discrete character evolution on the Maximum Clade Credibility tree from Kriebel et al. (2019).

Fig. S6. Results from OUwie analyses fitting evolutionary models to PC1 and PC2 of corolla, anther connective and style shape.