Greater pollination generalization is not associated with reduced constraints on corolla shape in Antillean plants

Simon Joly[1,2,*], François Lambert[2], Hermine Alexandre[2], Julien Clavel[3],
Étienne Léveillé-Bourret[2,**], and John L. Clark[4]

[1]*Montreal Botanical Garden, Montréal, Canada*
[2]*Institut de recherche en biologie végétale, Département de sciences biologiques, Université de Montréal, Montréal, Canada*
[3]*Institut de biologie, École Normale Supérieure, Paris, France*
[4]*Department of Biological Sciences, The University of Alabama, Tuscaloosa, USA*
[*]*To whom correspondance should be addressed: Institut de recherche en biologie végétale, 4101 Sherbrooke East, Montréal (QC) H1X 2B2, Canada; +1 514.872.0344; joly.simon@gmail.com.*
[**]*Present address: Department of Biology, University of Ottawa, Ottawa, Canada*

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Authors contributions

SJ conceived the study. FL, HA, ELB and JLC collected the data, SJ, FL and JC analyzed the data, SJ wrote the draft and all authors contributed and critically edited the final manuscript.

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Data availability

The data, phylogenetic trees, R scripts and interactive figures in html format are all available as supplementary material archive in Dryad (doi: xxx). Nucleotides sequences generated were deposited in GenBank.

Abstract

Flowers show important structural variation as reproductive organs but the evolutionary forces underlying this diversity are still poorly understood. In animal-pollinated species, flower shape is strongly fashioned by selection imposed by pollinators, which is expected to vary according to guilds of effective pollinators. Using the Antillean subtribe Gesneriinae (Gesneriaceae), we tested the hypothesis that pollination specialists pollinated by one functional type of pollinator have maintained more similar corolla shapes through time due to more constant and stronger selection constraints compared to species with more generalist pollination strategies. Using geometric morphometrics and evolutionary models, we showed that the corolla of hummingbird specialists, bat specialists, and species with a mixed-pollination strategy (pollinated by hummingbirds and bats; thus a more generalist strategy) have distinct shapes and that these shapes have evolved under evolutionary constraints. However, we did not find support for greater disparity in corolla shape of more generalist species. This could be because the corolla shape of more generalist species in subtribe Gesneriinae, which has evolved multiple times, is finely adapted to be effectively pollinated by both bats and hummingbirds. These results suggest that ecological generalization is not necessarily associated with relaxed selection constraints.

Key-words: Pollination syndromes, specialists-generalists, geometric morphometrics, phylogenetic comparative methods, Ornstein-Uhlenbeck (OU) models.

Introduction

The variation of flower shapes and structures we observe in nature is a constant reminder of the power of natural selection. This diversity is often attributed to zoophilous pollination, which has been associated with increased diversification in angiosperms (Stebbins, 1970; Crepet, 1984; Johnson, 2010; van der Niet and Johnson, 2012). Indeed, pollinator-driven selection pressure has been associated with species diversification (Whittall and Hodges, 2007), and frequent pollinator shifts often correlate with increased species diversification rates (e.g., Valente et al., 2012; Forest et al., 2014; Breitkopf et al., 2015). Yet, despite the numerous studies on pollination-driven selection at the population level (reviewed below), on the dissection of the genetic basis of several floral transitions between species pollinated by different pollinators (reviewed in: Galliot et al., 2006; Yuan et al., 2013) and of phylogenetic investigations of pollination systems at macroevolutionary levels (e.g., Perret et al., 2007; Smith et al., 2008), there is still a gap in our understanding on how the microevolutionary forces operating at the population level shape the macroevolutionary patterns we observe (Waser, 1998).

Selection can affect flower morphology differently when a population is adapting to a novel pollinator guild (transition phase) compared to when it is under the influence of a relatively constant pollinator guild (stasis phase). The transition phase is expected to involve strong directional selection until the population has a phenotype close to the optimum for the new pollinators (Lande, 1976). Studies on pollinator-mediated selection have found evidence for strong directional selection for flower shape in the transition phase (Galen, 1989), while others have shown that pollinators can drive flower colour transitions in populations (Waser and Price, 1981; Stanton et al., 1986). Although not a direct measurement of selection, the numerous studies reporting geographically structured flower variation associated with variation in pollinator guilds further support these findings (e.g., Gómez and Perfectti, 2010; Newman et al., 2014; Niet et al., 2014; Martén-Rodríguez et al., 2011), especially when reciprocal transplant experiments confirmed these patterns (Newman et al., 2012; Boberg et al., 2014; Sun et al., 2014).

For populations in stasis phase, that is with a relatively constant selection pressure from a stable pollinator guild, the floral traits are expected to be under stabilizing selection around optimal trait values. The mean phenotype of a population evolving under stabilizing selection is affected by both selection and drift, with selection pulling the mean phenotype towards the fitness optimum and drift due to finite population sizes moving it in random directions (Lande, 1976, 1979). Although stabilizing selection on floral traits have sometimes been observed in pollinator-mediated selection studies (Sahli and Conner, 2011; Conner et al., 2003), most studies failed to find such evidence (Campbell et al., 1991; O'Connell and Johnston, 1998; Maad, 2000). This might be because these phases are not so stable and that these studies are typically performed on a yearly basis. Indeed, studies have shown that selection on floral traits can vary from year to year in populations (Herrera, 1988; Campbell, 1989; Campbell et al., 1991) due to temporal variation in pollinator abundance or environmental conditions. Nevertheless, there is considerable evidence that traits involved in the mechanical fit between the flower and the pollinators are under long-term stabilizing selection pressure as they show less variation in populations than other traits (Muchhala, 2006; Cresswell, 1998). Interestingly, these observations suggest that evidence for such stabilizing selection might be better studied over many generations, or even at macroevolutionary scales, than for a single generation (see also Haller and Hendry, 2014).

The intensity of constraints during the stasis phase is also expected to vary according to the level of pollination generalization of the species of interest. If the flower shape of specialist flowers should show evidence of stabilizing selection around an optimal shape adapted to its pollinator, a greater diversity of processes can explain how pollinators affect selection of floral shape in generalists (Aigner, 2001, 2006; Sahli and Conner, 2011). The *trade-off* model suggests that a change in trait that increases the fitness contribution of one pollinator will decrease the fitness of another pollinator by a similar amount (Fig. 1; Aigner, 2001; Sahli and Conner, 2011). This scenario preditcs that generalist species will be morphologically intermediate between specialists. Moreover, because the fitness surface of generalists is flatter, this scenario predicts that generalists will tend to show relaxed selection constraints compared to specialists (Johnson and Steiner, 2000) and that generalists should show greater variation among populations or species than specialists, especially if pollinator abundance vary. An alternative model is the *trait specialization* that suggests that individual traits are under

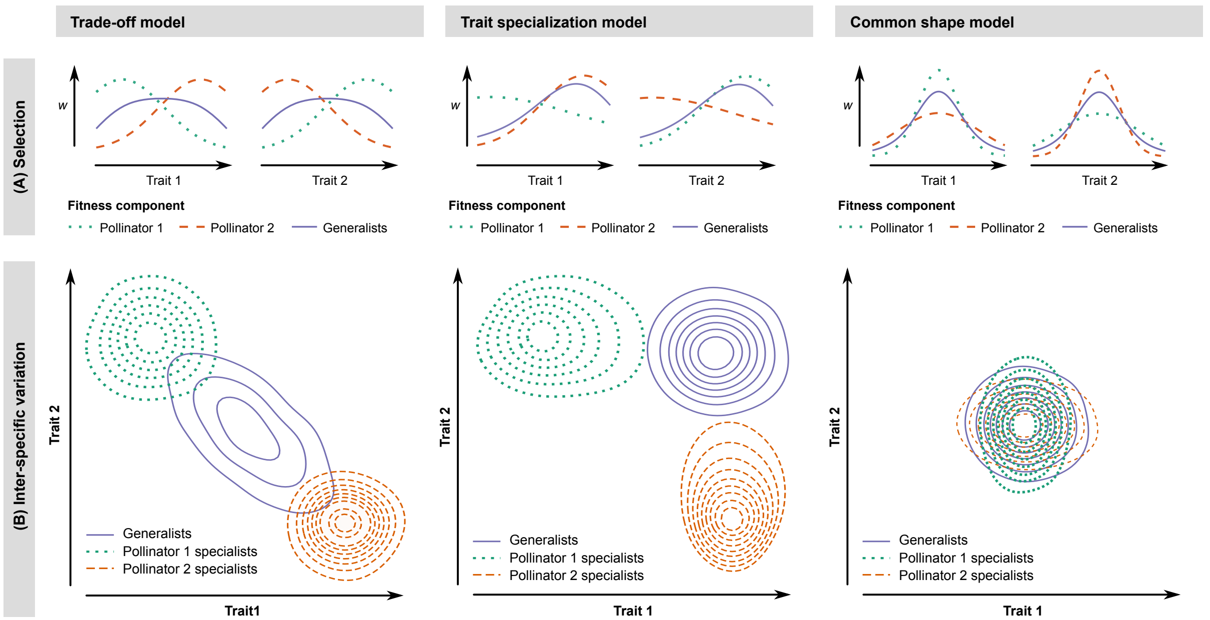

Figure 1: (A) Conceptual fitness functions describing how pollinators affect selection on floral trait in generalists for three distinct models. The dashed lines indicate selection by individual pollinators and the solid line the total selection on the traits of generalists when both pollinators are present in equal abundance (adapted from Sahli and Conner, 2011). (B) Expected densities of morphologies for specialists and generalists species for the three models. With the *trade-off* model, the generalists are intermediate in morphology between the two specialists and are expected to show greater morphological variation among species than specialists due to broader fitness function and possible fluctuating pollinator abundance. With the *trait specialization* model, the generalists occupy a distinct region of the morphological landscape and they do not (necessarily) show increased morphological variance compared to specialists.

selection by a subset of pollinators with none or a very asymetric trade-off (Fig. 1; see also Sahli and Conner, 2011). This could result in flowers that possess different traits adapted to different pollinators. With such a model, the expectation in terms of selection constraints and interspecific variation are similar for generalists and for specialists. Finally, the *common shape* model implies that the different pollinators all select for a common shape (Sahli and Conner, 2011). As for the *trait specialization* model, expectations in terms of selection and variation for generalists are similar to that for specialists under the *common shape* model.

In this study, we use a macroevolutionary approach to test expectations of the trade-off model on floral shape. Specifically, we test if increased generalization in pollination strategies is associated with relaxed selection constraints, or greater diversification (disparity), for corolla shape in species of the subtribe Gesneriinae of the Gesneriaceae family in the Caribbean islands. The recent development of powerful phylogenetic comparative methods allows the estimation of historic selective constraints on large groups of species (e.g., Hansen and Martins, 1996; Beaulieu et al., 2012; Butler and King, 2004) and thus testing specific hypotheses regarding the role of pollinators on floral trait evolution (Smith et al., 2008; Gómez et al., 2015; Lagomarsino et al., 2017). Unlike many investigations performed at the population level, such approaches aim at characterizing constraints on morphological variation over macroevolutionary scales and, as such, should be informative to understand the forces that have been determinant in modeling the morphology of large groups of species.

The subtribe Gesneriinae represents an ideal group to test this hypothesis. This diverse group in terms of floral morphologies is almost completely endemic to the Antilles and diversified into approximately 81 species (Skog, 2012) during the last 10 millions years (Roalson et al., 2008; Roalson and Roberts, 2016). The group has been the subject of several pollination studies that classified the species into different pollination syndromes that vary in their degree of ecological specialization (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009, 2010, 2015). There exists several definitions of pollination specialization/generalization, but globally plants pollinated by more species are considered more generalist (see papers in Waser and Ollerton, 2006), although information on the relative abundance (Medan et al., 2006) and functional diversity of pollinators (Johnson and Steiner, 2000; Fenster et al., 2004; Gómez and Zamora, 2006) should ideally be taken into account. Note that ecological generalization is completely independent of phenotypic specialization (Ollerton et al., 2007; Fleming and Muchhala, 2008; Armbruster, 2014); ecological generalists can be phenotypically specialized or not. Here, we follow Fleming and Muchhala (2008) and measure ecological specialization with respect to the number of effective functional pollinator groups, with species pollinated by more functional pollinator groups being more generalists.

Specialist pollination strategies in Gesneriinae include hummingbird pollination, bat pollination, moth pollination and bee pollination (Fig. 2). Species with these strategies are pollinated by a single functional type (or guild) of pollinator and most often by a single species (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009, 2010, 2015). A fifth pollination strategy is considered more generalist as it is effectively pollinated in similar proportions by hummingbirds and bats (Martén-Rodríguez et al., 2009), two pollinators belonging to different functional groups that have different plant growth form and floral

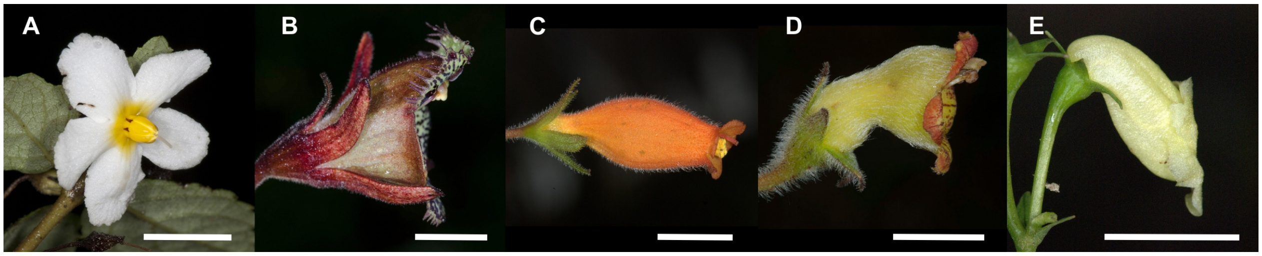

Figure 2: Gesneriinae flowers showing the different pollination strategies discussed in the study: (A) bee pollination (*Bellonia spinosa*, JLC 10573); (B) bat pollination (*Gesneria fruticosa*, JLC 14308); C) hummingbird pollination (*Rhytidophyllum rupincola*, JLC 11308 G4); D) mixed-pollination (*Rhytidophyllum auriculatum*, JLC 14499); E) moth pollination (*Gesneria humilis*, JLC 10574). The bar indicates 1 cm. Photographs by J.L. Clark.

(nectar, shape, colour) preferences (Baker, 1961; Faegri and van der Pijl, 1979; Flemming et al., 2005). Although there exists many examples of more generalist species, these species are nevertheless ecologically more generalized than species pollinated by a single functional group of pollinators because they rely on more diversified resources (Gómez and Zamora, 2006). To avoid any confusion, we will use the term mixed-pollination strategy to refer to them in this study. Species of the Gesneriinae are sometimes visited by insects, but these always have marginal importance (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009, 2015) except for the insect pollination strategies. A phylogenetic study of the group suggested multiple origins of most pollination strategies (Martén-Rodríguez et al., 2010), making it a perfect group to study selective forces acting on each one.

In this study, we augmented previous phylogenetic hypotheses of the group by adding more species and genetic markers and we used geometric morphometrics of corolla shape and evolutionary models to test that (1) corolla shape evolution in the group supports distinct pollination syndromes, (2) corolla shape evolution is characterized by long-term constraints, and that (3) the corolla shape of mixed-pollination species show greater disparity in floral shape compared to specialists.

Material and Methods

Floral morphology and pollination strategies

We collected photographs of 137 flowers in anthesis (137 distinct individuals, all from different localities) in longitudinal view, from 50 species (supplementary Table S1, S2; picture thumbnails are available as supplementary material) for a mean of 2.8 individuals per species (sd. dev. = 2.4). Most of these were taken in the wild, but a few specimens came from botanical gardens. We also took three pictures of the same flower (releasing and grabbing the pedicel between pictures) for four species at the Montreal Botanical Garden to quantify the error involved in hand-photographing the specimens as this is how most specimens were photographed.

Pollinator information was obtained from the literature (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009, 2010, 2015). Pollination strategy of species without field observation were inferred using the overall floral morphology following the conclusions of Martén-Rodríguez et al. (2009). Briefly, hummingbird specialists have straight tubular corollas with bright colours and diurnal anthesis, bat specialists have green or white campanulate (bell-shaped) corollas with nocturnal anthesis and exserted anthers, and species with a mixed-pollination strategy are intermediate with subcampanulate corollas (bell-shaped with a basal constriction) showing various colours with frequent coloured spots, and diurnal as well as nocturnal anther dehiscence and nectar production (Martén-Rodríguez et al., 2009, Fig. 1). So far, only one moth pollinated species has been observed and it has a pale pouched corolla (Fig. 1). All analyses were performed (1) using only species with confirmed pollinator information and (2) also adding species with inferred strategies. We followed the taxonomy of Skog (2012) except for recent modifications in the *Gesneria viridiflora* complex (Lambert et al., 2017).

Molecular methods

A total of 94 specimens were included in the phylogenetic analyses (supplementary Table S3). *Koehleria* sp. 'Trinidad' (tribe Gesnerieae) and *Henckelia malayana* (tribe Trichosporeae) were included as outgroups. DNA was extracted using the plant DNA extraction kits from QIAGEN (Toronto, Ontario) or BioBasics (Markham, Ontario). Five nuclear genes were amplified and sequenced: *CYCLOIDEA*, *CHI*, *UF3GT*, *F3H*, *GAPDH*. The first four are unlinked (unpublished linkage map), whereas no data is available for *GAPDH*. Primer sequences and PCR conditions can be found in supplementary Table S4. Sequencing reactions were performed by the Genome Quebec Innovation Centre and run on a 3730xl DNA Analyzer (Applied Biosystems). Sequences from both primers were assembled into contigs and corrected manually in Geneious vers. 1.8. DNA sequences generated for this study were augmented with previously published sequences (supplementary Table S3).

Phylogenetic analyses

Gene sequences were aligned using MAFFT (Katoh and Standley, 2013). Ambiguous alignment sections in intron regions of *CHI* and *GAPDH* were removed using gblocks (Castresana, 2000) with the default settings. Alignments were verified by eye and no obviously misaligned region remained after treatment with gblocks. Substitution models were selected by Akaike Information Criterion (AIC) with jModeltest 2 (Darriba et al., 2012) using an optimized maximum likelihood tree. A species tree was reconstructed using *BEAST in BEAST ver. 1.8.2 (Drummond et al., 2012). A Yule prior was chosen for the tree, a lognormal relaxed molecular clock for gene trees, and a gamma (2,1) prior for gene rates. Other parameters were left to the default settings. Three independent Markov Chain Monte Carlo (MCMC) analyses of 1×10^8 generations were performed, sampling trees and parameters every 10,000 generations. Convergence of the runs was reached for parameter values, tree topology and clade posterior probabilities. The first 2×10^7 generations were discarded as burnin and the remaining trees were combined for the analyses. The maximum clade credibility tree with median node heights was used for graphical representation.

Geometric morphometric analyses

Six landmarks and 26 semi-landmarks were positioned on photographs using `tpsDig2` (Rohlf, 2010) as in Alexandre et al. (2015). Two landmarks were positioned at the base of the corolla, two at the tips of the petal lobes, and two at the base of the petal lobes, which generally corresponds to the corolla tube opening. The semi-landmarks were then positioned at equal distance along the curve of the corolla (13 on each side) between the landmarks at the base of the corolla and at the base of the petal lobes. The sepals were present on most of the pictures. The landmark data was imported in `R` (R core team, 2014) where it was transformed by generalized Procrustes analysis using the `geomorph` `R` package (Adams and Otárola-Castillo, 2013). The semi-landmarks on curves were slid along their tangent directions during the superimposition by minimizing the Procrustes distance between the reference and target specimen (Bookstein, 1997). Size was not considered in the analyses because we were interested in shape and because a scale was not available for all specimens. Because the actinomorphic flowers of bee pollinated species (*Bellonia ssp.*) do not allow homologous placement of landmarks, these were removed from the morphometric analyses.

Landmarks were positioned twice for each photograph and a Procrustes ANOVA quantified the variance explained by these technical replicates, which were combined for the remaining analyses. We also used a Procrustes ANOVA to quantify the variation among the replicated photographs of the same flowers; these replicates were not included in the final analyses. The Procrustes aligned specimens were projected into the tangent space, hereafter the morphospace, using Principal Component Analysis (PCA) of the covariance matrix using the `prcomp` function in `R`.

To characterize the total morphological variation for each pollination strategy, we estimated the distance of the mean corolla shape of each species to the pollinator strategy centroid in multivariate space and tested if these distances were different for the different pollination strategies using the `betadisper` function of the `vegan` package in `R` (Oksanen et al., 2017). The differences were tested by ANOVA. We also partitioned the variation into intraspecific and interspecific components for each pollination strategy using Procrustes ANOVA, reporting adjusted R^2 values.

Morphological integration (Klingenberg, 2013) was quantified using the variance of the eigenvalues of a PCA on the covariance matrix (Pavlicev et al., 2009; Klingenberg, 2013), scaling the eigenvalues by the total variance of the sample to get an index independent of the total sample variation (Young, 2006). This was estimated on all individuals for the hummingbird and mixed-pollination species. Bat specialists were omitted from this analysis because there were too few species to give a result comparable to the other pollination strategies.

Ancestral states reconstruction

Ancestral state reconstruction was performed to estimate the probability of all pollination strategy states for all nodes of the phylogeny. The best transition model was first selected by second order AIC (AICc) with the `geiger` `R` package (Harmon et al., 2008). Eight models selected based on biological relevance were compared. The Equal Rate (ER), Symmetric (SYM) and All Rates Different (ARD) were tested with modified versions that give a single rate to and from the moth and bee states (ER.2, SYM.2, and ARD.2). In addition, a 4-rate model was tested where rates differed according to the actual state and a single rate to and from the bee and moth states, and finally a 3-rate model with one rate for transitions from and to bee and moth states, one from hummingbirds to bats or mixed-pollination, and a third from bat or mixed-pollination to all states except bee or moth. The bee and moth states were given the same rates in many models tested because they are both observed in a single species and it is thus difficult to accurately estimate rates to and from these states. Using the best model, the joint ancestral state probabilities were estimated using stochastic character mapping (Huelsenbeck et al., 2003) on the maximum clade credibility tree with 2000 simulated character histories. When estimating ancestral states with only species with confirmed pollinators, the other species were given equal prior probabilities in the simulations. To estimate the number of transitions between states while accounting for phylogenetic uncertainty, 500 character histories were simulated on 2000 species trees randomly sampled from the posterior distribution from the species tree search using the `phytools` `R` package. The median number of transitions between all states from all simulated character histories were reported as well as 95% credible intervals.

Evolutionary constraints on flower shape

Given the nature of the hypotheses tested, two types of evolutionary models based on the Brownian motion (BM) and the Ornstein-Uhlenbeck (OU) stochastic processes were considered. BM models the accumulation of independent and infinitesimal stochastic phenotypic changes (controlled by the drift rate parameter σ^2) along the branches of a phylogeny; it can approximate various scenarios of phenotypic evolution such as drift, fluctuating directional selection or punctuated change (Felsenstein, 1985; Hansen and Martins, 1996; O'Meara et al., 2006). In contrast, the OU process models selection toward a common optimal trait value (Felsenstein, 1988; Hansen and Martins, 1996) and adds to the BM model a selection parameter α that determines the strength of selection towards an optimal trait θ (details on the models can be found in Hansen and Martins, 1996; Butler and King, 2004; Beaulieu et al., 2012). When the strength of selection is null ($\alpha = 0$), the OU process reduces to BM. These models can be made more complex, for instance by allowing parameters to vary in different parts of the tree (selective regimes - e.g., Butler and King, 2004; O'Meara et al., 2006; Beaulieu et al., 2012) and are therefore useful for characterizing the evolutionary constraints of the pollination strategies.

The OU and BM models can thus be useful to test the presence of selective constraints on traits. However, BM and OU processes can be difficult to distinguish, and an OU process can best fit the data for other reasons such as measurement error (Silvestro et al., 2015), bounded trait variation (Boucher and Démery, 2016) or small sample sizes (Cooper et al., 2016). In contrast, OU models are less likely to be selected when analyzing the primary axes of variation from a PCA (Uyeda et al., 2015). Therefore, prediction of selective constraints are often better assessed through evaluation of parameters estimated under OU or BM.

According to the *trade-off* model, generalist species should exhibit greater phenotypic disparity of corolla shape because they are thought to be under weaker selection (Johnson and Steiner, 2000) and because of the spatio-temporal variation in pollinator abundance that could result in fluctuating selection pressures (Herrera, 1988). This prediction can thus be evaluated by looking at the parameter estimates of the BM and OU models. With the BM process, the drift rate (σ^2) describes the accumulation of phenotypic variance over the tree and is therefore tightly related to phenotypic disparity (O'Meara et al., 2006; Thomas et al., 2006;

Price et al., 2013). Following our hypothesis of lower phenotypic disparity for pollination specialists, we predict they should have a smaller σ^2 compared to mixed-pollination species. Similarly, under an OU model, the stationary variance around an optimum, expressed as $\sigma^2/2\alpha$ for the univariate case, is also tightly related to phenotypic disparity. We thus expect pollination specialists to be associated with stronger corolla shape constraints (i.e., higher α values) and smaller stationary variances compared to mixed-pollination species. Finally, we expect phenotypic evolutionary correlations between traits inferred from multivariate comparative models to be higher in pollination specialists (i.e., higher phenotypic integration, see for instance Revell and Collar, 2009) as this is also suggestive of stronger constraints.

We evaluated and compared the model fit and parameter estimates with the predictions of our hypotheses using univariate and multivariate models because they allow investigating different aspects of the data. Univariate models allowed us to fit a greater range of evolutionary models that are not yet implemented in multivariate approaches and allow investigating if different shape components evolved under similar constraints. In contrast, multivariate models allow to fit an evolutionary model on several shape components at once and also allow to investigate patterns of evolutionary correlations among traits for the different pollination strategies; that is, studying phenotypic integration in an evolutionary context.

For univariate models, we fitted BM models with one drift rate for the whole tree (BM1) and with one rate per regime (BMV), but also versions that allow different ancestral states for the different regimes (O'Meara et al., 2006; Thomas et al., 2009); model BM1m has distinct trait means per regime but a single drift rate across the tree, while BMVm has distinct means and drift rates for each regime. We also fitted different variants of the OU models (Beaulieu et al., 2012): with a single optimum θ (OU1), with different optima for lineages with different pollination strategies (OUM), different θ and selective strength α (OUMA), different θ and rates of stochastic motion σ^2 (OUMV), or different θ, α and σ^2 (model OUMVA) for the different pollination strategies. We also considered ecological release models, in which one regime on the tree is evolving under BM and the other under an OU process, either with a shared drift rate σ^2 (OUBM and BMOU) or with their own drift rates (models OUBMi and BMOUi) which are sometimes called ecological release and radiate models (see Slater, 2013). The model OUBM considers hummingbird specialists to be evolving under an OU model whereas the mixed-pollination species are evolving under a BM model, and vice versa. Several multivariate models were also considered: BM1, BMV, BM1m, BMVm, OU1, OUM, OUBM, BMOU, OUBMi, and BMOUi. The multivariate OU models allowing different contraints on different regimes (OUMA, OUMV, OUMVA) are not implemented yet and thus we can not estimate regime specific evolutionary covariance (or correlation) matrices. However, we expect such models to be over-parameterized with respect to the number of species considered in our study.

We compared the phenotypic evolutionary correlations obtained from the σ^2 correlation matrices of the multivariate BM models for the different pollination strategies. Yet, focussing on the interpretation of parameters obtained under the BM processes can be misleading if BM is a poor descriptor of the phenotypic evolution (see for instance Price et al., 2013). To make sure this did not affect our estimates, we simulated datasets using a OUM model on 100 trees randomly selected from the posterior distribution using the parameters estimated from the observed data. We then fitted these simulated data with the BMVm model to obtain σ^2 correlation matrices that were compared with the original σ^2 correlation matrices.

The models were fitted for the first three principal components of the morphospace using the `R` packages `mvMORPH` (Clavel et al., 2015) and `OUwie` (Beaulieu et al., 2012). The models were fitted on a sample of 1000 trees from the posterior distribution of species trees on which the character history was inferred by one instance of stochastic mapping (Huelsenbeck et al., 2003) using maximum likelihood in the `phytools R` package (Revell, 2012). This accounts for phylogenetic uncertainty and the stochasticity of the character state reconstructions (Revell, 2013). All the trees were re-scaled to unit height. Intraspecific variation was taken into account by using the sampling variance (the squared standard error) of species as measurement error in model fitting; species without biological replicates were given the mean squared standard error of species with the same pollination strategy. The models were compared using *AICc* weights that can be roughly considered as the relative weight of evidence in favour of a model given a set of models (Burnham and Anderson, 2002). The analyses were performed with inferred pollination strategies as well as with species with confirmed pollination strategies only. Note that because there were few confirmed bat pollinated species and a single moth pollinated species, species with these pollination strategies were excluded from the analyses. However, the inclusion of bat pollinated species in the univariate models did not affect the conclusions (data not shown). The data and scripts used to replicate all analyses are available as supplementary information.

Results

Phylogeny

The species phylogeny suggested that the bee pollinated genus *Bellonia* is sister to the rest of the subtribe, and the subtribe (*Bellonia* + *Gesneria* + *Rhytidophyllum*) received a posterior probability of 1 (not shown). *Rhytidophyllum* and *Gesneria* were found to form distinct clades, although *Gesneria* received weaker support (Fig. 3). This reinforces the distinction between these two genera, which has been debated over the years. There is one exception, *Rhytidophyllum bicolor*, which is included for the first time in a molecular phylogeny and that falls within *Gesneria*. The taxonomic name of this species will have to be reconsidered. Several branches show strong clade posterior probabilities, but some had less support due to lack of phylogenetic signal or conflict between gene trees, indicating the importance of incorporating phylogenetic uncertainty in the following analyses.

The best character evolutionary model (smallest $AICc$) for ancestral states reconstruction was the 3 rates model with one rate for transitions from and to the bee and moth states, one from hummingbirds to bats or mixed-pollination, and a third from bat or mixed-pollination to all states except bee and moth. Ancestral state reconstruction (Fig. 3) suggests that the hummingbird pollination is the most likely ancestral state for the *Gesneria* clade, although it is only slightly more likely than an ancestral mixed-pollination strategy. In contrast, the mixed-pollination strategy is the most probable ancestral state for the *Rhytidophyllum* clade. A hummingbird pollinated ancestor for the subtribe is more probable, but only very slightly. This reflects the difficulty in estimating the ancestral states for nodes near the root of a phylogeny (Gascuel and Steel, 2014). The ancestral state reconstruction with the inferred pollination strategies (Fig. S1) were highly similar to those of Fig. 3.

Estimation of the number of transitions supports several transitions between the bat, the mixed-pollination and the hummingbird strategies (Table 1). The number of transitions from

Table 1: Number of transitions between the different pollination strategies according to the stochastic mapping. The median values obtained from the character simulations over the posterior distribution of species tree are reported as well as 95% credible intervals. Ancestral states are in rows.

	bat	bee	hummingbird	mixed-pollination	moth
bat	–	0.30 [0.22, 0.37]	3.31 [2.79, 3.88]	3.52 [3.10, 3.91]	0.26 [0.17, 0.31]
bee	0.07 [0.02, 0.12]	–	0.07 [0.03, 0.11]	0.08 [0.03, 0.14]	0.04 [0.03, 0.07]
hummingbird	2.61 [2.16, 3.03]	0.61 [0.52, 0.71]	–	2.52 [2.10, 2.89]	0.84 [0.71, 0.98]
mixed-pollination	4.30 [3.68, 4.77]	0.36 [0.29, 0.43]	4.87 [4.14, 5.51]	–	0.31 [0.21, 0.37]
moth	0.04 [0.02, 0.06]	0.08 [0.04, 0.11]	0.05 [0.03, 0.08]	0.04 [0.02, 0.07]	–

mixed-pollination to hummingbird and from mixed-pollination to bat was slightly higher than from bat to mixed-pollination and bat to hummingbird, which was also slightly higher than the number of transitions from hummingbird to bats and hummingbird to mixed-pollination (Table 1). However, because the confidence intervals largely overlap, we can conclude that the number of transitions between these three main pollination strategies are not significantly different. The results were almost identical when analyses were performed with inferred pollination strategies (Supplementary Table S5). These estimates are similar to those of Martén-Rodríguez et al. (2010), although they found fewer reversals to hummingbirds in their study. Overall, these results confirm multiple evolutionary origins for all pollination strategies except for the bee and moth (95 % CI always > 2; Table 1).

Corolla shape

We found only 0.15% of variation between independent pictures of the same flower in the replication experiment, which is lower than the variation involved in the landmark positioning (0.81%). Therefore, we conclude that the error included in the data by the picture acquisition was minimal. Similarly, because the technical replicates accounted for only 0.56% of the total variance in the final dataset, the mean shape between replicates was used for the remaining analyses.

The morphospace explained 79% of the total shape variance in the first three axes. The first principal component (PC) represents 53.6% of the variance and is characterized by campanulate (bell-shaped) vs. tubular corollas (Fig. 4A), broadly differentiating hummingbird specialists from the other species. This concurs with a previous study that showed that this was indeed the main characteristic differentiating the hummingbird pollination strategy from the bat and the mixed-pollination strategies (Martén-Rodríguez et al., 2009). PC2 explains

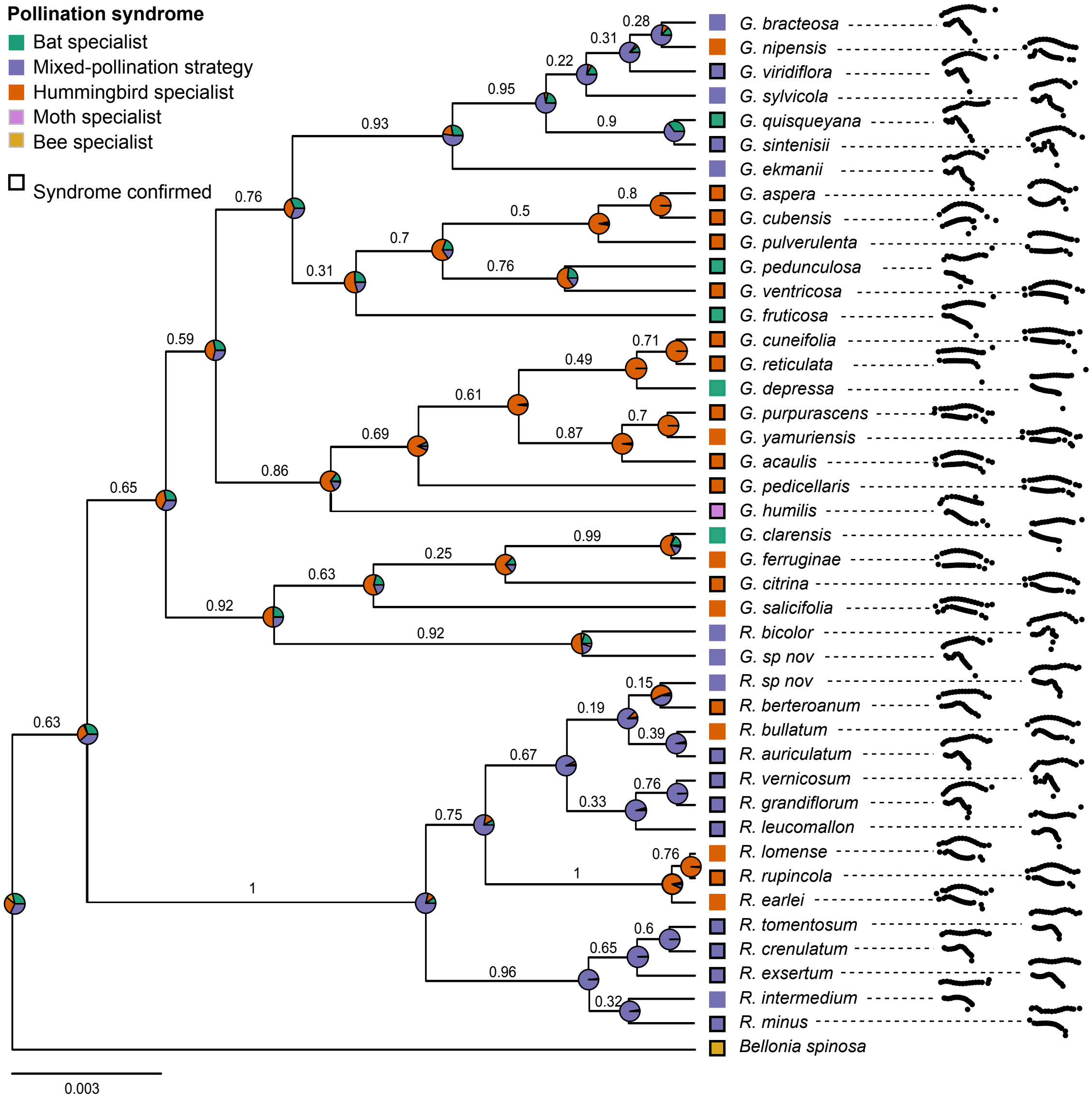

Figure 3: Species phylogeny showing mean corolla shapes (after Procrustes analysis). Pollination strategies are shown with those that have been confirmed indicated by a black contour. Pie charts represent the joint probability of each state at nodes as estimated by stochastic mapping from only species with confirmed pollinators. Clade posterior probabilities are shown above branches. Outgroup taxa are not shown.

20.6% of the variance and is characterized by corolla curvature and distinguished the moth pollinated *G. humilis*. The bat and the mixed-pollination strategies could not be differentiated with this PCA, but a second PCA that excluded moth and hummingbird pollinated species (both confirmed and inferred) found that the bat and mixed-pollination strategies were separated along PC3 that is characterized by a basal constriction in the corolla (Fig. 4B), a character known to distinguish bat pollinated species (that generally lack the constriction) from species with a mixed-pollination strategy (Martén-Rodríguez et al., 2009). The single bat pollinated species that groups with mixed-pollination species on this axis is *Gesneria quisqueyana* (see interactive supplementary figures S2 and S3 for information on the individual and species positioning in the PCAs), which, in contrast to other bat pollinated species in the group, excludes hummingbirds during the day by actively closing its flowers (Martén-Rodríguez et al., 2009).

Variation partitioning

The pollination strategies did not have a significantly different corolla variation among species (ANOVA: F = 1.92, df = 2, $p = 0.1654$). The partitioning of the shape variance for the different pollination strategies showed that the proportion of variance explained among species corresponded to 81.4% ($p < 0.001$) for hummingbird pollinated species, 91.3% ($p = 0.22$) for bat pollinated species and 50.4% ($p < 0.001$) for mixed-pollination species. The result of the variance partitioning for the bat pollinated species should be interpreted with caution because there were only three species with less than two replicated individuals on average within species for this strategy.

Morphological integration

Flower components are generally well integrated as they develop, function and evolve jointly (Ashman and Majetic, 2006), a concept called morphological integration (reviewed in Klingenberg, 2013). A large morphological integration index supports important integration because morphological variation is concentrated in few principal components. The results showed that species with a mixed-pollination strategy had a slightly greater morphological

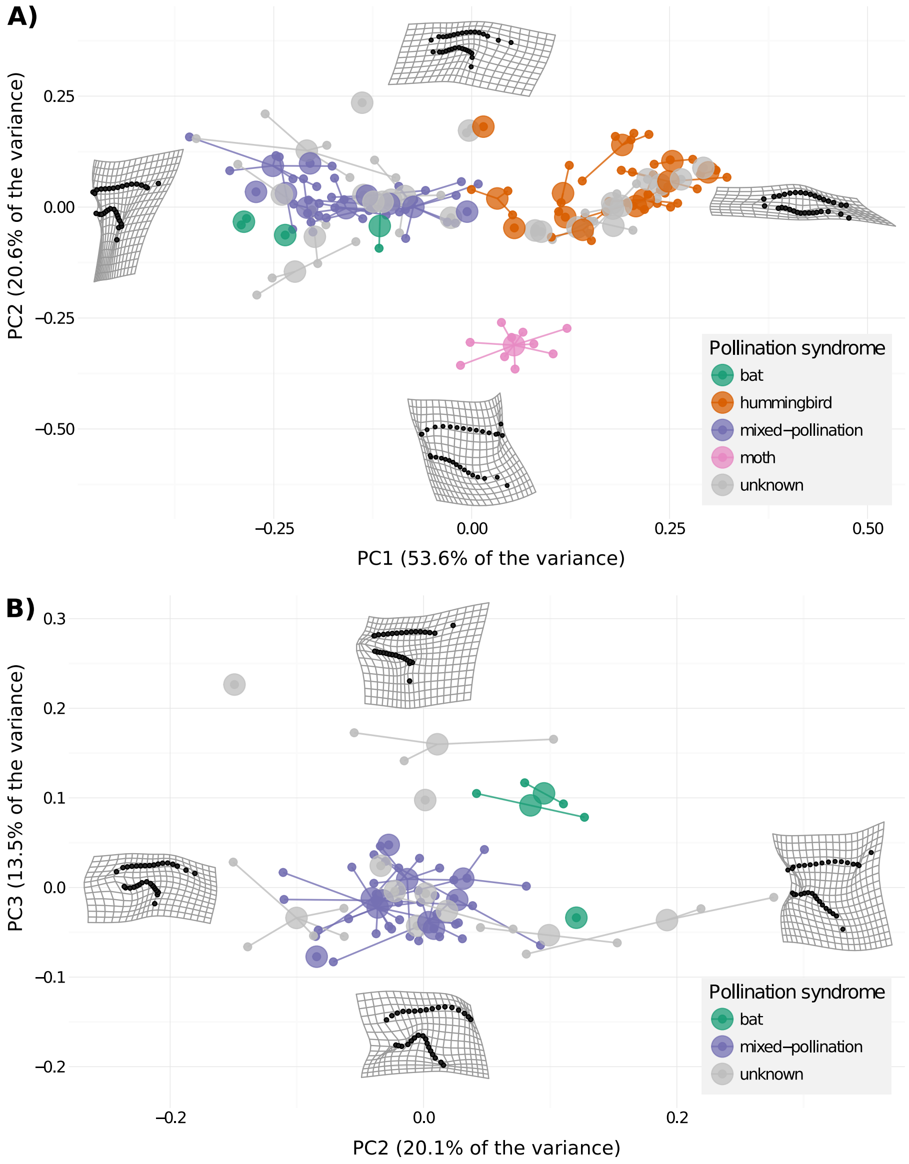

Figure 4: Corolla shape morphospaces obtained from principal component analyses performed on (A) all species or on (B) species excluding hummingbird (both confirmed and inferred) and moth pollinated species. The large dots on the plot represent the species means, which are connected by a line to the floral shapes of the individuals belonging to the species (small dots). Thin-plate spline deformation grids show corolla shape variation along the principal components (plus or minus 2 standard deviation from the mean shape). *Bellonia spinosa* (bee pollinated) was not included in the morphometric analyses because it has a radial symmetry.

integration (0.0069) than hummingbird pollinated species (0.0050).

Evolutionary models

Univariate models

For PC1 that captures variation in corolla opening, all models that received $AICc$ weights greater than zero suggest that the hummingbird specialists and the mixed-pollination species differed in their mean shape as they all included distinct θ for the two strategies (Table 2). The best models, OUM and BM1m ($AICc$ weight of 0.48 and 0.35, respectively), suggest that the two pollination strategies had similar evolutionary phenotypic variance as they constrain them to have identical parameters. This trend is also supported by parameter estimates of supported models that allowed the strategies to differ in drift rates (BMVm) or stationary variance (OUMV, OUMA, OUMVA) as these estimates were very similar for the two strategies (Table 2). The phylogenetic half-life of the OUM model, which corresponds to the time required for the expected phenotype to move half-way towards the optimal shape from its ancestral state (Hansen, 1997), was 0.009. Given that the trees were scaled to unit height, this small value imply either strong selective pressure (see Hansen et al., 2008) or a lack of phylogenetic correlation. The results of the analyses that included species with inferred pollination strategies were very similar in terms of model selection and phenotypic disparity (Table S6).

The PC2 of the morphospace that represents variation in the curve of the corolla was found to best fit a OUBMi model ($AICc$ weight = 0.72; Table 2), with the hummingbird pollinated species evolving under a OU model and the mixed-pollination species evolving under a BM model, each with their own drift rate implying that this model cannot be simply interpreted as reduced constraints for mixed-pollination species. Nevertheless, the model suggests that the pollination strategies have the same mean shape for PC2 and that the two pollination strategies have evolved under different types of constraints. The median phylogenetic half-life was of 0.02 for the hummingbird species, suggesting either strong selective pressure or a lack of phylogenetic correlation. Parameter estimates for the other models, in particular the second best model OUMV ($AICc$ weight = 0.15), also supported similar mean shapes for

Table 2: Parameter values of the univariate evolutionary models fitted on the first three principal components of the morphospace when only the species with confirmed pollinators were included in the analyses. Mean values from the posterior distribution of species trees are given for the AICc weights, whereas median values are given for the parameter estimates. Numbers in brackets indicate the 25% and the 75% quantiles. The best model for each PC is in bold. The θ parameter indicate the global or regime means (ancestral states) for the BM-type and OUBM-type models, whereas it indicates the stationary optimum trait for the OU-type models. $station_{hum}$ and $station_{mix}$ are the stationary variance of the hummingbird and mixed-pollination strategies.

PC1

Models	p	$AICc$ weight	θ_{hum}	θ_{mix}	σ^2_{hum}	σ^2_{mix}	$station_{hum}$	$station_{mix}$
BM1	2	0 [0,0]	0.057 [0.05,0.066]	0.057 [0.05,0.066]	0.046 [0.037,0.074]	0.046 [0.037,0.074]	–	–
BMV	3	0 [0,0]	0.07 [0.011,0.113]	0.07 [0.011,0.113]	0.035 [0.02,0.093]	0.045 [0.026,0.086]	–	–
BM1m	3	0.35 [0.02,0.68]	0.15 [0.144,0.155]	-0.123 [-0.131,-0.112]	0.017 [0.013,0.023]	0.017 [0.013,0.023]	–	–
BMVm	4	0.1 [0.01,0.17]	0.146 [0.138,0.153]	-0.124 [-0.132,-0.112]	0.021 [0.014,0.031]	0.013 [0.012,0.016]	–	–
OU1	3	0 [0,0]	0.05 [0.039,0.057]	0.05 [0.039,0.057]	0.088 [0.061,0.197]	0.088 [0.061,0.197]	0.029 [0.029,0.031]	0.029 [0.029,0.031]
OUM	**4**	**0.48 [0.11,0.84]**	**0.16 [0.152,0.163]**	**-0.155 [-0.156,-0.152]**	**0.859 [0.265,3.004]**	**0.859 [0.265,3.004]**	**0.006 [0.006,0.006]**	**0.006 [0.006,0.006]**
OUMV	5	0.05 [0,0]	0.197 [0.18,0.222]	-0.134 [-0.226,-0.094]	8.248 [3.772,23.45]	7.442 [2.802,21.646]	2.684 [1.381,5.179]	2.696 [1.443,4.635]
OUMA	5	0.02 [0,0]	0.195 [0.178,0.23]	-0.14 [-0.246,-0.09]	8.935 [3.876,19.47]	8.935 [3.876,19.47]	2.573 [1.857,3.386]	2.667 [2.107,3.739]
OUMVA	6	0.01 [0,0]	0.195 [0.175,0.212]	-0.141 [-0.257,-0.106]	7.756 [3.12,23.395]	6.743 [2.518,18.899]	2.439 [1.388,4.782]	3.018 [1.516,4.646]
OUBMi	4	0 [0,0]	0.125 [0.106,0.142]	0.125 [0.106,0.142]	0.252 [0.065,4.186]	0.042 [0.023,0.064]	0.009 [0.007,0.013]	–
BMOUi	4	0 [0,0]	0.038 [-0.03,0.092]	0.038 [-0.03,0.092]	0.04 [0.021,0.095]	0.06 [0.032,0.104]	–	460558 [0.017,1.82×10^8]
OUBM	3	0 [0,0]	0.135 [0.12,0.151]	0.135 [0.12,0.151]	0.073 [0.056,0.13]	0.073 [0.056,0.13]	0.007 [0.006,0.009]	–
BMOU	3	0 [0,0]	0.043 [-0.045,0.059]	0.043 [-0.045,0.059]	0.053 [0.037,0.107]	0.053 [0.037,0.107]	–	18437 [0.009,5.55×10^7]

PC2

Models	p	$AICc$ weight	θ_{hum}	θ_{mix}	σ^2_{hum}	σ^2_{mix}	$station_{hum}$	$station_{mix}$
BM1	2	0 [0,0]	-0.035 [-0.037,-0.032]	-0.035 [-0.037,-0.032]	0.021 [0.013,0.038]	0.021 [0.013,0.038]	–	–
BMV	3	0.04 [0,0.04]	-0.035 [-0.041,-0.025]	-0.035 [-0.041,-0.025]	0.036 [0.021,0.065]	0 [0,0]	–	–
BM1m	3	0 [0,0]	-0.031 [-0.035,-0.028]	-0.041 [-0.048,-0.036]	0.021 [0.013,0.038]	0.021 [0.013,0.038]	–	–
BMVm	4	0.01 [0,0.01]	-0.028 [-0.036,-0.02]	-0.038 [-0.046,-0.026]	0.036 [0.02,0.064]	0 [0,0]	–	–
OU1	3	0.01 [0,0.02]	-0.036 [-0.036,-0.036]	-0.036 [-0.036,-0.036]	0.972 [0.18,1.104]	0.972 [0.18,1.104]	0.003 [0.003,0.003]	0.003 [0.003,0.003]
OUM	4	0 [0,0.01]	-0.042 [-0.044,-0.042]	-0.027 [-0.027,-0.026]	0.499 [0.107,1.041]	0.499 [0.107,1.041]	0.003 [0.003,0.003]	0.003 [0.003,0.003]
OUMV	5	0.15 [0.01,0.28]	-0.026 [-0.039,-0.014]	-0.017 [-0.04,-0.01]	10.239 [5.932,18.219]	0.018 [0,0.072]	1.663 [1.314,2.512]	0.003 [0,0.01]
OUMA	5	0 [0,0]	-0.024 [-0.04,-0.015]	-0.016 [-0.038,-0.005]	6.319 [3.531,11.251]	6.319 [3.531,11.251]	0.868 [0.671,1.145]	0.868 [0.671,1.145]
OUMVA	6	0.02 [0,0.03]	-0.025 [-0.038,-0.013]	-0.017 [-0.041,-0.011]	13.96 [6.714,26.595]	0.027 [0.001,1.106]	1.772 [1.316,3.017]	0.004 [0,0.167]
OUBMi	**4**	**0.72 [0.53,0.93]**	**-0.043 [-0.045,-0.041]**	**-0.043 [-0.045,-0.041]**	**0.305 [0.132,0.93]**	**0 [0,0]**	**0.004 [0.004,0.004]**	–
BMOUi	4	0.01 [0,0.01]	-0.035 [-0.041,-0.025]	-0.035 [-0.041,-0.025]	0.036 [0.021,0.065]	0.001 [0,0.001]	–	0 [0,0]
OUBM	3	0 [0,0]	-0.044 [-0.05,-0.035]	-0.044 [-0.05,-0.035]	0.03 [0.019,0.064]	0.03 [0.019,0.064]	0.003 [0.003,0.005]	–
BMOU	3	0.03 [0,0.02]	-0.029 [-0.038,-0.025]	-0.029 [-0.038,-0.025]	0.036 [0.021,0.064]	0.036 [0.021,0.064]	–	0 [0,0]

PC3

Models	p	$AICc$ weight	θ_{hum}	θ_{mix}	σ^2_{hum}	σ^2_{mix}	$station_{hum}$	$station_{mix}$
BM1	2	0.06 [0.01,0.09]	0.017 [0.015,0.019]	0.017 [0.015,0.019]	0.005 [0.004,0.006]	0.005 [0.004,0.006]	–	–
BMV	3	0.02 [0,0.03]	0.017 [0.015,0.019]	0.017 [0.015,0.019]	0.005 [0.004,0.006]	0.005 [0.004,0.006]	–	–
BM1m	3	0.02 [0,0.03]	0.023 [0.022,0.026]	0.004 [0.001,0.007]	0.005 [0.004,0.005]	0.005 [0.004,0.005]	–	–
BMVm	4	0.01 [0,0.01]	0.024 [0.022,0.026]	0.004 [0.001,0.007]	0.005 [0.004,0.006]	0.005 [0.004,0.006]	–	–
OU1	3	0.18 [0.04,0.27]	0.013 [0.013,0.016]	0.013 [0.013,0.016]	0.036 [0.017,0.739]	0.036 [0.017,0.739]	0.002 [0.002,0.002]	0.002 [0.002,0.002]
OUM	4	0.05 [0.01,0.07]	0.015 [0.014,0.019]	0.012 [0.011,0.013]	0.03 [0.017,0.713]	0.03 [0.017,0.713]	0.002 [0.002,0.002]	0.002 [0.002,0.002]
OUMV	**5**	**0.36 [0.18,0.53]**	**0.027 [0.025,0.029]**	**0.014 [0.01,0.022]**	**13.351 [7.115,26.325]**	**3.692 [2.121,6.126]**	**1.033 [0.744,1.621]**	**0.291 [0.215,0.359]**
OUMA	5	0.12 [0.04,0.17]	0.026 [0.022,0.028]	0.017 [0.01,0.024]	4.885 [3.11,13.095]	4.885 [3.11,13.095]	0.505 [0.395,0.728]	0.505 [0.395,0.728]
OUMVA	6	0.04 [0.02,0.06]	0.027 [0.024,0.029]	0.014 [0.009,0.022]	6.412 [4.403,19.508]	2.492 [1.504,5.957]	0.734 [0.556,1.073]	0.264 [0.196,0.375]
OUBMi	4	0.01 [0,0.01]	0.015 [0.013,0.018]	0.015 [0.013,0.018]	0.012 [0.009,0.021]	0.005 [0.004,0.006]	0.002 [0.002,0.003]	–
BMOUi	4	0.04 [0.01,0.04]	0.015 [0.014,0.017]	0.015 [0.014,0.017]	0.005 [0.004,0.006]	0.041 [0.021,0.233]	–	0.001 [0.001,0.001]
OUBM	3	0.03 [0,0.04]	0.017 [0.014,0.02]	0.017 [0.014,0.02]	0.007 [0.006,0.009]	0.007 [0.006,0.009]	0.003 [0.002,0.004]	–
BMOU	3	0.07 [0.01,0.07]	0.015 [0.013,0.017]	0.015 [0.013,0.017]	0.006 [0.005,0.008]	0.006 [0.005,0.008]	–	0.001 [0.001,0.001]

the two pollination strategies and suggest that hummingbird pollinated species have greater phenotypic disparity as they have a greater stationary variance than mixed-pollination species (Table 2). The median phylogenetic half-life for the OUMV model was estimated to be 0.23, suggesting moderate constraints on corolla shape. The analyses including species with inferred pollination strategies best supported a OU1 model ($AICc$ weights = 0.69; Table S6) indicating a lack of evidence for different constraints or disparity for the two strategies. But, the total variance accumulated for each strategies under the OUMV and OUMVA models was higher for hummingbird pollinated species than for mixed-pollinated species (Table S6).

The PC3 that represents variation in the reflexion of the petal lobes (not shown) was found to best fit a OUMV model ($AICc$ weight = 0.36), although models OU1 and OUMA also received considerable weights ($AICc$ weights of 0.18 and 0.12, respectively; Table 2). All three models suggest that this shape component tends to stay closer to the evolutionary mean than would be expected under a BM model. The OU1 suggests that the pollination strategies have the same mean shape, whereas the OUM and OUMV models suggest different mean shapes, although parameter estimates for these later models showed that the mean shapes for both strategies are not very far from each other (Table 2). The models OUMV and OUMA suggest different shape disparity with the hummingbird specialists having a higher stationary variance than mixed-pollination species. The models OUMV, OU1 and OUMA all suggested strong constraints with estimated phylogenetic half-lifes of 0.11, 0.08, and 0.14, respectively. In analyses with species with inferred pollination strategies, the OU1 model received the highest weight (0.30), although several models received weights greater than 0.05 (Table S6). As for the analyses with only species with confirmed pollination strategies, the hummingbird pollinated species showed higher stationary variance in models in which this parameter was allowed to vary between strategies (Table S6).

In some instances, the models OU1 and OUM did not always converge to the maximum likelihood solution when fitted with `OUwie`, especially for PC1. This is why we always fitted these models with `mvMORPH`, which is also faster. Similarly, the models OUMV, OUMA, and OUMVA showed relatively poor convergence and should be interpreted with caution.

Multivariate models

The multivariate analyses supported OUM as the best fitting model ($AICc$ weight = 0.60; Table 3). This model suggests that the shape components have different evolutionary means for the two pollination strategies and that there is an evolutionary force that maintains the corolla shape closer to this evolutionary mean than would be expected under a BM model. The shape means estimated under the multivariate OUM model for each PC were very similar to that of the univariate estimates, as were the estimates of the stationary variance and phylogenetic half-lifes (compare Tables 2 and 4). The stationary variance estimates were also similar to the observed variance among species for hummingbird pollinated species (PC1: 0.0068, PC2: 0.0049, PC3: 0.0041) and mixed-pollinated species (PC1: 0.0075, PC2: 0.0014, PC3: 0.0016), suggesting that the model is very close to be stationary.

Because the current implementation do not allow the estimation of regime-specific evolutionary correlations between traits under the multivariate OUM model, we looked at the evolutionary correlations (σ^2_{ij} between traits i and j) under the BMVm model, which was the third best supported model ($AICc$ weight = 0.13; Table 3), to estimate the morphological integration for the two pollination strategies. The evolutionary correlations between shape components were always greater for the mixed-pollination strategy in terms of absolute correlation, although there is some uncertainty in these estimates as evident from the 50% confidence intervals estimated over posterior distribution of trees (Fig. 5). Furthermore, the better support for the OUM and BM1m models also suggests that differences between pollination strategies are probably marginal or that we lack statistical power to detect significant differences. Because these correlations were obtained on a BMVm model whereas a OUM model was the one that received the highest support, there is a risk that the younger mixed-pollination clades may appear to have evolved faster under the BMVm model (Price et al., 2013), which could in turn affect the observed correlations. However, this does not seem to be the case as the correlations estimated on data simulated with the OUM model were similar between pollination strategies (Fig. 5), rejecting the possibility that the greater absolute correlations observed for the mixed-pollination strategy were due to model misspecification. The multivariate results obtained when species with inferred pollinators were included were similar, with even more support for the OUM model ($AICc$ weight = 1; Ta-

Table 3: Model performance with the multivariate evolutionary models fitted on the first three principal components of the morphospace when only confirmed species are included in the analyses. The mean values obtained from the posterior distribution of species trees are given; numbers in brackets indicate the 25% and the 75% quantiles. The best model is in bold.

Models	*logLik*	Parameters	*AICc* weight
BM1	67.98 [63.43,76.25]	9	0.00 [0.00,0.00]
BMV	80.44 [76.94,86.18]	15	0.00 [0.00,0.00]
BM1m	78.52 [72.7,86.95]	12	0.24 [0.00,0.45]
BMVm	90.47 [85.96,96.59]	18	0.13 [0.00,0.12]
OU1	82.30 [74.47,87.04]	15	0.02 [0.00,0.00]
OUM	**96.24 [93.47,98.18]**	**18**	**0.60 [0.03,1.00]**
OUBM	77.17 [74.14,82.47]	15	0.00 [0.00,0.00]
BMOU	80.47 [76.97,85.85]	15	0.01 [0.00,0.00]
OUBMi	95.24 [93.99,97.05]	21	0.01 [0.00,0.00]
BMOUi	83.71 [80.01,89.11]	21	0.00 [0.00,0.00]

Table 4: Model parameters for the multivariate OUM model, which was the model that received the highest *AICc* weight (Table 3). The mean values obtained from the posterior distribution of species trees are given; numbers in brackets indicate the 25% and the 75% quantiles. The complete stationary variance-covariance matrix is given in Table S7.

parameters	PC1	PC2	PC3
θ_{hum}	0.161 [0.152,0.166]	-0.043 [-0.046,-0.042]	0.013 [0.009,0.015]
θ_{mix}	-0.156 [-0.159,-0.154]	-0.026 [-0.027,-0.023]	0.013 [0.012,0.02]
σ^2	1.198 [0.135,0.135]	1.328 [0.184,0.184]	0.757 [0.005,0.005]
Phylogenetic half-life	0.002 [0.001,0.003]	0.01 [0.003,0.031]	0.101 [0.01,0.194]
Stationary variance	0.006 [0.005,0.006]	0.003 [0.003,0.003]	0.002 [0.002,0.002]

bles S8, S9). However, the correlation between traits suggest phenotypic integration of more similar amplitude for the two pollination strategies with inferred pollinators (Fig. S2). The discrepancies between the results with all species and only those with confirmed pollination strategies could be due to the small size of the datasets as such correlations are difficult to estimate accurately.

Discussion

Although many aspects of the flower are required for assuring successful reproduction, the corolla shape is critical for the adaptation of plants to pollinators. In many species, the corolla guides the pollinator to allow precise pollen deposition on its body (Muchhala, 2007). But pollinators can also show an inherent preference for some floral shapes (Gómez et al., 2008) and can associate shape and reward when these are correlated (Meléndez-Ackerman et al., 1997). Floral shape has in fact repeatedly been shown to be under selection in pollination-

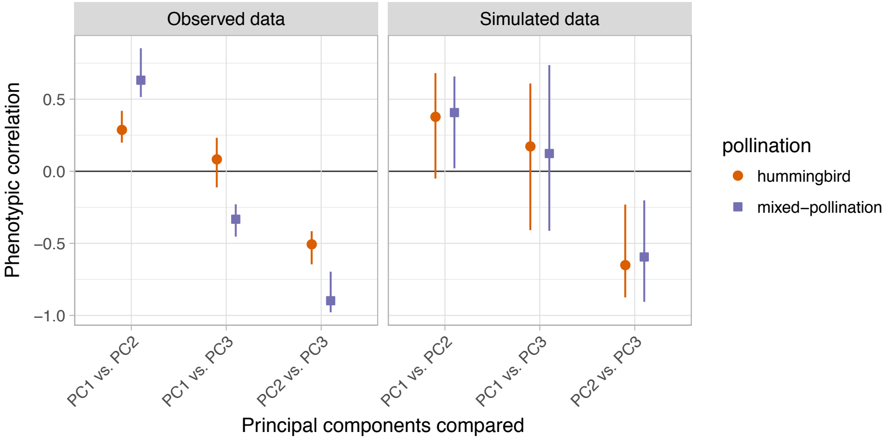

Figure 5: Graphical representation of the evolutionary correlations (from standardized evolutionary rates matrices) obtained with the BMVm multivariate model with only species with confirmed pollination strategies, for the observed data (left panel) and for data simulated under the best fitting model (OUM; right panel). Symbols represent the median correlation and the lines the 25% and 75% quantiles for both hummingbirds and mixed-pollination strategies. No artifactual differences are detected between the two groups when fitting models on traits simulated with the OUM model and thus with a common evolutionary covariance (right panel, see text).

driven selection studies (Galen, 1989; Campbell et al., 1991; O'Connell and Johnston, 1998; Maad, 2000) and can be sufficient to impose adaptive trade-off between pollinators (Muchhala, 2007). Even the corolla shape of highly generalist species has been shown to adapt to particular guilds of pollinators (Gómez and Perfectti, 2010; Gómez et al., 2015).

In the Antillean genera *Gesneria* and *Rhytidophyllum*, pollination syndromes are well characterized and have good predictive value (Martén-Rodríguez et al., 2009), but previous studies were based on attractive and mechanical floral characters. Our results, based on geometric morphometrics alone, showed that it is possible to distinguish corollas of hummingbird pollinated species and moth pollinated species, and, although to a lesser degree, the corolla shapes of species with bat or mixed-pollination strategies. These conclusions were reinforced by the strong support in favour of distinct shapes for hummingbird specialists and mixed-pollination species in evolutionary models, both based on parameter estimates and on support for models supporting different evolutionary shape means (BMm models) or distinct shape optima (OUM models). These results, in addition to the fact that each pollination strategy evolved repeatedly in the Gesneriinae, further support the concept of pollination syndromes in this group and underlines the importance of corolla shape in floral adaptation to pollinators.

Long-term evolutionary constraints on corolla shape

In this study, we wanted to test if the corolla shape of flowers have evolved under evolutionary contraints to maintain effective pollination and to test the expectations of the *trade-off* model that the floral shapes of the more generalist species should show greater morphological disparity (Fig. 1).

All analyses performed, both univariate and multivariate and using only species with confirmed pollinator information or also including species with inferred strategies, selected OU models that possess an α parameter that maintains the corolla shape closer to an evolutionary optimum than expected under a BM model. This supports the hypothesis that the corolla shape in the group has been affected by long-term evolutionary constraints, which could be interpreted as a consequence of the selective pressure imposed by pollinators. The analyses found very small phylogenetic half-lifes that are suggestive of very strong selection pressures and/or lack of phylogenetic correlation in the data. Considering a potential origin of the group ca. 10 mya (Roalson et al., 2008; Roalson and Roberts, 2016) and taking the smallest phylogenetic half-life obtained (0.002, for the PC1 in the multivariate analysis; Table 4), this means that a corolla shape can move half-way to its optimal shape in $0.002 \times 10 = 0.02$ million years, or 20,000 years. This is rapid, but not impossible considering that transitions between pollination strategies are generally driven by few genes of major effects (Galliot et al., 2006; Yuan et al., 2013), implying that such changes can occur rapidly on macroevolutionary scales. Because floral shape in the group is under the control of a small number of loci of moderate effects (Alexandre et al., 2015), a rapid evolution seems more likely than a simple lack of phylogenetic signal.

Contrarily to the predictions of the *trade-off* model, we did not find evidence that more generalist mixed-pollination species accumulated greater phenotypic disparity compared to specialists. The non-phylogenetic approaches suggested similar amount of variation among species for both pollination strategies, and this pattern was confirmed by the evolutionary models. Indeed, almost all analyses selected a model in which both strategies evolved under shared constraints, but for different means for each selective regime. Moreover, although the differences were marginal, the parameter estimates of the evolutionary models that allows the two strategies to have different phenotypic disparities almost constantly indicated that it was the hummingbird specialists that showed a higher disparity compared to the more generalist mixed-pollination species.

Morphological integration and evolutionary correlations between shape components allows us to take another view at evolutionary contraints on corolla shape. Indeed, important integration between the shape components suggests tight coordination for proper functioning and strong evolutionary correlations suggest that components have evolved in an highly coordinated fashion. The results showed both higher morphological integration and evolutionary correlations for the mixed-pollination species, which goes against the prediction of the *trade-off* model that more generalist species are less constrained. Overall, we come to the conclusion that greater generalization in pollination strategies did not imply a relaxation of evolutionary constraints over macroevolutionary scales in Antillean Gesneriinae.

The fact that all lines of evidence do not suggest relaxed contraints on the floral shape of mixed-pollination species argues against the *trade-off* model in terms of how pollinators affect selection on traits and alternative models need to be considered. Clearly, the *common shape* model is not compatible with this group because the results clearly showed that the different pollinators favor distinct floral shapes. In contrast, the data seem to better fit the *trait specialization* model given that the mixed-pollination species do not show greater floral shape disparity than the specialists and have a distinct floral shape that appears to be optimized for pollination by both bats and hummingbirds. Indeed, it has been proposed that the presence of a constriction at the base of the corolla for species with a mixed-pollination strategy could represent an adaptation to allow a good pollination service by both hummingbirds and bats by forcing them to approach the flower in a specific way (Martén-Rodríguez et al., 2009). The fact that the corolla shape typical of this pollination strategy has evolved recurrently in the group (Fig. 3) certainly adds weight to this hypothesis. These mixed-pollination species might thus have a phenotypically specialized corolla, in the sense that it is well adapted to both bat and hummingbird pollination, even though they are ecological generalists by being pollinated by different functional pollinators. Indeed, concepts of phenotypic specialization and ecological specialization need not be correlated (Ollerton et al., 2007; Fleming and Muchhala, 2008; Armbruster, 2014). This strategy might be particularly successful in fine-grained pollination environment (Aigner, 2006), such as where pollinators are scarce or vary through time (Waser et al., 1996). Such hypothesis of adaptive generalization (see Gómez and Zamora, 2006) certainly deserves more attention in the future, and will require information on pollination frequency and efficiency to properly associate flower shape to the relative efficiency of pollinators.

The detection of selection contraints for both pollination strategies is noteworthy given that several factors probably contribute in reducing this signal over macroevolutionary time scales. For instance, temporal variation in pollination guilds over macroevolutionary times could weaken the signal of selection, mirroring observations at the population level (e.g., Campbell, 1989; Campbell et al., 1991). The pollination guilds were assumed to be functionally constant over time in our analyses, but given that the exact species pollinating the flowers vary among plant species (Martén-Rodríguez et al., 2009, 2015), the whole story might be more complex. For instance, unrecognized sub-syndromes could be responsible for the larger variation observed for the hummingbird strategy and additional pollinator information will be needed to investigate this further. Variation in selective pressure among species could also occur if agents other than pollinators affect corolla shape. For instance, the apical constriction of the corolla of hummingbird pollinated *Drymonia* (Gesneriaceae) has recently been suggested to be an adaptation to exclude bees (Clark et al., 2015). Moreover, herbivores, including nectar robbers, may affect the selective forces imposed on flowers by pollinators (e.g., Galen and Cuba, 2001; Gómez, 2003). While non-pollinating floral visitors–including bees–are generally not abundant in the group (Martén-Rodríguez et al., 2009, 2015) and herbivory is not common (pers. obs.), it is difficult to completely discard this possibility.

This study showed evidence of constrained evolution on flower shapes imposed by pollinator guilds over macroevolutionary time scales and as such demonstrated the usefulness of a phylogenetic approach to understand pollinator mediated selection. Although additional investigations are needed to confirm these patterns, this study certainly adds weight to the evidence accumulated by many others over the years that the specialist - generalist continuum in pollination biology is complex (Waser et al., 1996; Waser and Ollerton, 2006) and that we cannot assume a priori that pollination specialists show reduced phenotypic disparity compared to pollination generalists.

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