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Pollinator specialization imposes stronger evolutionary constraints on flower shape

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Abstract

Flowers show an unrivalled diversity as reproductive organs but the evolutionary forces underlying this diversity are still poorly understood. In animal-pollinated species, flower shape is fashioned by selection imposed by pollinators, which is expected to vary according to specific guilds of effective pollinators. Using the Antillean subtribe Gesneriinae (Gesneriaceae), we tested the hypothesis that the corolla shapes of specialists effectively pollinated by one functional type of pollinator have maintained more similar shapes through time due to stronger selection constraints than those of species effectively pollinated by more than one functional type of pollinator. Using geometric morphometrics, we show that corolla shape can differentiate hummingbird specialists, bat specialists, and species with a mixed-pollination strategy (pollinated by hummingbirds, bats, and occasionally insects). Then, using evolutionary models, we show that the corolla shape of hummingbird specialists has been evolving under balancing selection, whereas a neutral model of evolution was favoured for mixed-pollination species. This suggests that the corolla shape of pollination specialists remains more similar over macro-evolutionary periods of time to remain fitted to their pollinators. In contrast, corollas of species with a mixed-pollination and thus more generalized strategy vary more, potentially because they experience effective pollination over a wider corolla shape space.

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

Introduction

The astonishing diversity of shape and colour of angiosperm flowers are a great demonstration of the role of natural selection in modelling the morphology of organisms. This diversity is often attributed to zoophilous pollination that provides a wealth of reproduction strategies (Stebbins, 1970), but we still know little of the underlying evolutionary forces (Waser, 1998). An important step towards understanding floral diversity is to characterize and quantify the evolutionary forces acting on floral characters (Rosas-Guerrero et al., 2014).

In animal-pollinated species, flowers are fashioned by selection imposed by pollinators, which is expected to vary according to the guild of effective pollinators (Faegri and van der Pijl, 1979; Fenster et al., 2004; Rosas-Guerrero et al., 2014). In this context, a useful concept is that of pollination syndrome, which represents the combination of pollinator-related traits to which flowers of different species pollinated by functionally similar pollinators converge (Faegri and van der Pijl, 1979; Fenster et al., 2004; Rosas-Guerrero et al., 2014). For instance, hummingbird pollinated flowers typically have red flowers with a tube-shaped corolla (Fenster et al., 2004). Although this concept has sometimes been disputed (e.g., Ollerton et al., 2009), it generally holds when applied to groups of functionally equivalent pollinators (Rosas-Guerrero et al., 2014). Pollination syndromes imply that floral evolution is the result of natural selection that acts to enhance pollen deposition on the stigmatic surface of conspecific flowers (Martén-Rodríguez et al., 2009). As such, floral traits should be driven primarily by the most effective pollinators (Stebbins, 1970) and potentially also by counter-effective pollinators via trade-off effects (Aigner, 2001; Armbruster, 2014).

Flower traits involved in pollination are often conveniently classified as "attractive" traits (Ashman and Morgan, 2004) or "mechanical fit" traits (Cresswell, 1998). Attractive traits are those involved in pollinator attraction such as flower colour, nectar volume and composition, and fragrance. In contrast, mechanical fit traits mostly consist of flower shape traits and are thought to be mostly associated with the transfer of the pollen to the stigma. These mechanical fit traits are expected to be particularly affected by pollinators as selection is expected to favour traits that have the highest overall fitness for a given guild of pollinators (Aigner, 2001).

The consequences of pollinator-imposed selection on flower shape are easy to predict on specialist flowers that are pollinated by one species, or by a functional equivalent set of pollinators. That is, flower shape of specialist flowers should show evidence of balancing

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selection around an optimal shape adapted to its functional pollinators. In contrast, the expectations of selective pressure on flower shape are less clear for generalist flowers that can be effectively pollinated by several pollinators (Aigner, 2001; Sahli and Conner, 2011). In general, unless the different functional pollinators all select for a common shape (common peak model: Sahli and Conner, 2011), generalists effectively pollinated by more than one functional type of pollinators are expected to be under weaker selection pressure (Johnson and Steiner, 2000) than specialists.

This prediction does not seem to have been tested thoroughly even if these questions are important to understand how and why flowers diversify under the selection of animal pollinators (Johnson, 2010). Insights on these questions are likely to be gained through both microevolutionary and macroevolutionary approaches (Johnson, 2010). There is considerable evidence that traits involved in the mechanical fit between the flower and the pollinators are under stronger selective pressure as they show less variation in populations (e.g., Muchhala, 2006; Cresswell, 1998) and because their parts show greater integration (Ashman and Majetic, 2006). There is also much evidence that flower shape of both specialist and generalist flowers is affected by the relative abundance of their pollinators over their species ranges (e.g., Gómez and Perfectti, 2010; Newman et al., 2014; Niet et al., 2014; Martén-Rodríguez et al., 2011) and at the macroevolutionary level (e.g., Gómez et al., 2015). However, few studies have contrasted the selective forces acting on flowers for different levels of pollination generalization for a given group of plants.

In this study, we use a macroevolutionary approach to test whether increased specialism in pollination is associated with a stronger selection pressure on corolla shapes of species of the subtribe Gesneriinae of the Gesneriaceae family in the Caribbean islands. The recent development of powerful phylogenetic comparative methods allow estimating historic selection pressures on large groups of species (Beaulieu et al., 2012; Butler and King, 2004) and thus testing specific hypotheses regarding the role of pollinators on floral trait evolution (e.g., Gómez et al., 2015). 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). With one exception, the species can be classified into three main pollination syndromes: hummingbird specialists, bat specialists, and species with a mixed-pollination strategy. Species of the latter category are effectively pollinated by combinations of hummingbirds, bats and, occasionally, insects (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009, 2010). Although these are probably not true generalists, they are nevertheless less specialized than species of syndromes adapted to a single functional set of pollinators. A phylogenetic study of the group has suggested that the hummingbird syndrome is probably the ancestral state in the group and that there has

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been multiple shifts towards bat and mixed-pollination strategies (Martén-Rodríguez et al., 2010). In this study, we use evolutionary models and geometric morphometrics to test (1) if flower shape as characterized by geometric morphometrics can distinguish the different pollination syndromes, and (2) if specialists evolved under stronger balancing selection compared to mixed-pollination species.

Material and Methods

Floral morphology and pollination syndromes

We took 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). Most of these were taken in the wild, but a few specimens came from botanical gardens. We also photographed three times the same flower (releasing the flower between each) for four different species at the Montreal Botanical Garden to quantify potential error in hand-photographed specimens, as this is how most of the specimens were photographed in the wild.

Pollination syndrome information per species were obtained from the literature (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., 2009, 2010) and inferred for species with clear hummingbird and bat syndromes as these syndromes can be predicted perfectly (Martén-Rodríguez et al., 2009). Because this is not the case for species with a mixed-pollination syndrome, these were not predicted. Analyses were repeated with only species with confirmed pollination syndromes. In the Antillean subtribe Gesneriinae, hummingbird specialists have a tubular corolla with bright colours (yellow, orange or red) and diurnal anthesis, bat specialists have a campanulate (bell-shaped) corolla of light colours (green or white) and a nocturnal anthesis, and species with a mixed-pollination strategy are intermediate with a subcampanulate corolla (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). We followed the current taxonomy (Skog, 2012), although we consider subspecies *viridiflora*, *sintenisii*, *quisqueyana* and *acrochordonanthe* of *Gesneria viridiflora* to be distinct species (F. Lambert et al., unpublished data). It should be noted that species referred to as having a mixed-pollination syndrome were previously termed generalists, but we prefer the term mixed-pollination as these species are generally effectively pollinated by only two functional sets of pollinators.

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Molecular methods

A total of 94 specimens were included in the phylogenetic analyses (supplementary Table S3). The species *Koehleria '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 (H. Alexandre, unpublished data), whereas no data is available for *GAPDH*. Primer sequences and PCR conditions can be found in supplementary Table S4. PCR reactions included 1 $\times$ buffer, 1 mM $MgSO_4$, 1 U DreamTaq (Thermoscientific), 0.4 μM of each primer, 0.2 μM of each dNTPs, 1% PVP (M.W. 40,000), 50 μg BSA and ca. 30 ng of DNA. 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 regions in *CHI* and *GAPDH* due to introns 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. The best substitution models were selected by Aikake 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 vers. 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 and the trees and parameters were sampled 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

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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 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). This analysis translates specimens to the origin, scales them to unit-centroid size, and rotates them using a least-squares criterion until the coordinates of corresponding points align as closely as possible (Klingenberg, 2010). 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 proper scale was not available for all specimens.

Each photograph was numerized twice and a Procrustes ANOVA was performed to quantify the variance explained by the technical replicates in the whole dataset. These replicates 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 using Principal Component Analysis (PCA) of the covariance matrix. The PCA scores represent the shape variables that were used in the following analyses.

To characterize the total morphological variation for each pollination syndrome, we estimated the distance of the mean corolla shape of each species to the pollinator syndrome centroid in multivariate space and tested if these distances were different for the different syndromes. This was done using the `betadisper` function of the `vegan` package in `R` and the differences were tested by ANOVA. We also partitioned the variation into intraspecific and interspecific components for each pollination syndrome using Procrustes ANOVA, reporting adjusted R^2 values.

Phylogenetic comparative analyses

Ancestral pollinators were estimated at all nodes of the phylogeny except for the root as such estimates are generally unreliable (Gascuel and Steel, 2014). The best transition model was first selected by AIC with the `geiger` `R` package. Five models were compared based on biological relevance: Equal Rate (ER), Symmetric (SYM), All Rates Different (ARD), a 3 rates model where rates differed according to the actual state, and a 2 rates model with a distinct rate for transitions from hummingbirds to other syndromes. Using the best model, the joint ancestral state probabilities were estimated using stochastic character mapping (Huelsenbeck et al., 2003). Two thousand character histories were simulated on the maximum clade credibility tree using the `phytools` `R` package. The joint probabilities of observing each state at each node were then estimated. The number of transitions between states was counted by attributing to each node the state with maximum joint probability and counting

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the number of each type of transition on all branches of the phylogeny (except between the root and its daughter clades). The same approach was also performed on 5000 trees sampled from the posterior distribution of trees to get credible intervals around the estimates.

To test if corolla shape has been evolving under balancing selection, we fitted Brownian motion (BM) and Ornstein-Uhlenbeck (OU) models on corolla shapes of hummingbird specialists and species with a mixed-pollination strategy. There were too few bat specialists to properly estimate the fit of these models. The BM model is a standard null model in evolutionary biology and it assumes that evolution proceeds randomly along the branches of the tree without any selection pressure. The OU model differs from the BM by the presence of an optimal shape (θ) and a parameter (α) that determines the strength of selection (Butler and King, 2004). For a single quantitative character X evolving according to a OU model, the change in character value over the infinitesimal time interval between t and $t + dt$ is defined as $dX(t) = \alpha[\theta - X(t)]dt + \sigma dB(t)$, where $dB(t)$ is the rate of evolution with parameter σ giving its strength (Butler and King, 2004). For a multivariate definition of the models, see (Bartoszek et al., 2012). It is easy to see that when $\alpha = 0$, the model becomes equivalent to the BM model: $dX(t) = \sigma dB(t)$.

Genetic studies of pollination syndrome transitions in *Rhytidophyllum* (Gesneriaceae; Alexandre et al., 2015) and in other species (reviewed in Galliot et al., 2006) suggest that few genomic regions of moderate importance are generally involved in flower shape modifications. This suggests that transitions occur rapidly, almost instantaneously in a macro-evolutionary time frame. Consequently, evolutionary models that fit multiple regimes on a tree (e.g., Butler and King, 2004) are not appropriate because they assume that change is gradual. To circumvent this issue, we used a censored approach (O'Meara et al., 2006) and pruned the phylogeny to keep only species of a given syndrome at a time, and fitted the BM and OU models separately for the different syndromes. This approach allows us to focus on the selection pressures on corolla shape for a given pollination syndrome, that is once the morphological transition has occurred, and ignore the selection pressure involved in the transitions.

We accounted for intraspecific variation when fitting the model by including the standard error as measurement error for each species (Silvestro et al., 2015). Species without biological replicates were given the mean standard error of species with the same pollination syndrome. We also incorporated phylogenetic uncertainty by fitting the models on 5000 randomly sampled species trees from the posterior distribution. For each tree, the BM and OU models were fitted for hummingbird and mixed-pollination syndrome data and the models were compared by AIC. Replicates for which convergence was not attained were removed. We fitted the data using univariate and multivariate approaches (i.e., fitting two principal components simultaneously) using the `mvMORPH` `R` package (Clavel, 2014). The first two principal components of the morphospace, which represent 75 % of the total shape variance, were considered.

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To further validate the results, we also performed the model comparisons using only species with confirmed pollinator syndromes and also with species from a single pollination syndrome origin. The data and scripts used are available as supplementary information.

Results

Phylogeny

The species phylogeny shows that the genus *Bellonia*, with a bee pollination syndrome (Martén-Rodríguez et al., 2009), has a basal position in the subtribe and that *Rhytidophyllum* and *Gesneria* form two distinct clades, although *Gesneria* is less well supported (Fig. 1). This reinforces the distinction between these two genera, which has been debated over the years. There is one exception, *Rhytidophyllum bicolor*, which is incorporated in a molecular phylogeny for the first time and that falls within the *Gesneria* clade, and the status of this species will have to be re-evaluated. Several branches show strong clade posterior probabilities, but some less so due to lack of phylogenetic signal or conflict between genes trees.

The best evolutionary model (smallest AICc) was the two-rates model with one rate for the transition between either mixed-pollination or bat syndromes to hummingbird, and another from the hummingbird syndrome to either the bat or the mixed-pollination syndromes. Ancestral state reconstruction by maximum likelihood suggests that the hummingbird syndrome is the most likely ancestral state for both *Gesneria* and *Rhytidophyllum*, supporting previous results that this is the ancestral state in the group (Martén-Rodríguez et al., 2010). However, because these nodes are deep in the phylogeny, it is difficult to obtain very strong support for a hummingbird-pollinated ancestor, especially given that *Bellonia spinosa*, the sister-group, is bee pollinated (not included in the reconstruction). Estimation of the number of transitions between syndromes supports several transitions between most syndromes, although the most frequent transitions are from hummingbird to mixed-pollination (4), from hummingbird to bat (2), and from mixed-pollination to hummingbird (2) (Table 1). These results support the presence of reversals to the ancestral hummingbird pollination, but not definitively as the 95% CI includes 0 (Table 1).

Corolla shape

We found that only 0.15% of variation was explained 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 is minimal. Similarly, the technical replicates (landmark positioning)

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accounted for only 0.56% of the total variance in the complete dataset. Consequently, the mean shape per individual was used for the remaining analyses.

The projection of the landmarks into the tangent space by mean of PCA, hereafter named the morphospace, shows that the hummingbird specialists can be differentiated from the rest of the species using corolla shape (Fig. 2A). These groups are differentiated by the first principal component that characterizes floral opening (Fig. 2A), which represents the main characteristic thought to differentiate the hummingbird pollination syndrome from the bat and the mixed-pollination syndromes (Martén-Rodríguez et al., 2009). The bat and the mixed-pollination syndromes could not be differentiated with this PCA, but a second PCA that excluded species with a hummingbird syndrome found that the bat and mixed-pollination syndrome where somewhat separated along the third principal component (Fig. 2B). The only bat pollinated species that groups with mixed-pollination species on this axis is *Gesneria quisqueyana* (see supplementary figures S1 and S2 for information on the individual and species positioning in the PCAs), which, in contrast to other bat pollinated species in the group, excludes hummingbird during the day by actively closing its flowers (Martén-Rodríguez et al., 2009). This might explain the odd positioning of this species in the morphospace. Interestingly, the third principal component that contributes in distinguishing species with bat and mixed-pollination syndromes is characterized by the presence of a corolla constriction (Fig. 2B), which generally distinguishes bat pollinated species (that generally lack the constriction) and species with a mixed-pollination strategy (Martén-Rodríguez et al., 2009).

Variation partitioning

The pollination syndromes 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 syndromes 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 would be interpreted with caution though because there were few replicated individuals within species for this syndrome.

Evolutionary models

To test if corolla shape has been evolving under balancing selection, we fitted OU and BM models on the posterior distribution of species trees and compared their fit by AIC. Because a smaller AIC value is better, a positive difference in AIC between the simpler and the

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more complex models ($\Delta AIC = AIC_{BM} - AIC_{OU}$) supports the OU model, whereas a value equal or below 0 supports the simpler BM model. The distribution of ΔAIC for species with a mixed-pollination strategy included 0 in its 95% credible interval (CI) for the multivariate models (Table 2), thus rejecting the OU model. In contrast, the distribution of ΔAIC for hummingbird specialists is positive and the 95% CI excluded 0 in the multivariate model (Table 2), supporting the OU model and the presence of balancing selection in the evolution of corolla shape for hummingbird specialists. With the univariate models, the OU model was rejected for both pollination syndromes for PC1, but as for the multivariate analyses, the OU model was favoured for PC2 for the hummingbird syndrome but not for the mixed-pollination syndrome (Table 2). The results were identical when only species with experimentally confirmed syndromes or from a single evolutionary origin were analyzed (supplementary Fig. S3).

Discussion

Flowers, as reproductive organs, are more varied than equivalent structures of any other group of organisms (Barrett, 2002). Although many aspects of the flower are required for assuring successful reproduction, corolla shape is critical for the adaptation of plants to pollinators. Pollinators often show an inherent preference for some floral shapes (Gómez et al., 2008) and can associate shape and reward when these are correlated (Melédez-Ackerman et al., 1997), resulting in flower shape being often the target of natural selection. Moreover, it has been shown that floral shape alone is sufficient to impose an adaptive trade-off between hummingbird and bat pollination (Muchhala, 2007). Even the corolla shape of generalists can adapt to particular guilds of pollinators (Gómez and Perfectti, 2010; Gómez et al., 2015).

In the Antillean genera *Gesneria* and *Rhytidophyllum*, we were first interested to test whether pollination syndromes could be identified by corolla shape alone. Pollination syndromes are well characterized and have good predictive value in this group (Martén-Rodríguez et al., 2009), but previous studies were based on both attractive and mechanical floral characters. Our results based on geometric morphometrics show that it is possible to distinguish corolla shapes of hummingbird pollinated species, and, although to a lesser degree, the corolla shapes of species with bat or mixed-pollination syndromes. This supports the concept of pollination syndromes, especially since all syndromes have more than one origin in the Gesneriinae (Fig. 1; Table 1), and it reinforces the evolutionary importance of corolla shape in this group.

It is possible that the analysis of corolla shape using three-dimensional geometric morphometrics (van der Niet et al., 2010) could have allowed a finer characterization of flower shapes and could have allowed a finer delimitation of pollination syndromes. However, scan-

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ning flowers in three dimensions requires fresh material, which is not available for the current group as the vast majority of species are not found in gardens. In lieu of a 3D approach, the present results show the power of geometric morphometrics for studying floral shapes. Indeed, geometric morphometric methods are especially insightful for studying the associations between floral shape variation and pollination biology because they provide better shape characterization than simple linear measurements and by their capacity to easily separate size and shape aspects of floral variation (van der Niet et al., 2010). Supporting this, a recent study on the genetic basis of flower shape variation in *Rhytidophyllum* has shown that geometric morphometrics allowed a more thorough shape characterization than simple traits (Alexandre et al., 2015).

Selective forces acting on floral shape

We found that the different pollination syndromes had relatively similar overall variation, a pattern that can be observed when inspecting the dispersion of individuals on the morphospace (Fig. 2A) and confirmed by the distance-based variance analyses including all shape dimensions. This might be suggestive of similar selective pressure on flower shape for the different pollination syndromes, but reaching such a conclusion would ignore the distinct evolutionary histories of the syndromes. Indeed, because the pattern observed amongst living species represents the end result of evolution in groups that have diverged for different amounts of time, it is important to use a phylogenetic approach to quantify selection pressure at macro-evolutionary scales.

Based on previous studies that estimated selection pressures on floral traits for specialists and generalists at the intraspecific level (Muchhala, 2006; Gómez and Perfectti, 2010; Cresswell, 1998), we hypothesized that evidence for balancing selection on flower shape of specialists might also be detectable at macro-evolutionary scales, and that its strength would be greater than for species with a mixed-pollination strategy. We used a censored approach to quantify balancing selection within each pollination syndrome using an Ornstein-Uhlenbeck (OU) model and tested evidence for selection by comparing this model to a simpler neutral model of evolution (Brownian motion). The idea behind our approach was to quantify a potential macroevolutionary selective pressure within pollination syndromes that would work to pull corollas toward an optimal shape, while random mutation processes adds morphological variance in the model. Of course, we acknowledge that other types of selection might have been important as well. For instance, there was certainly directional selection occurring during pollination syndrome transitions. However, our censored approach should remove the influence of selection that is acting during pollination syndrome transitions. Selection could also have shaped floral morphology of some species, either via selection by specific pollinator species or because of interspecific competition for pollinators. However, these latter selective

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forces should be (mostly) independent among lineages and thus should not mask the major macroevolutionary trends.

We found a clear support for the OU model for describing the flower shape evolution of hummingbird pollinated species, which suggests the presence of an optimal corolla shape for these species and a balancing selection pressure that works to maintain corolla shapes close to its optimal shape, resulting in a relative morphological stasis in a macro-evolutionary time frame. In contrast, no such selection pressure could be detected in species with a mixed-pollination strategy. These findings support our initial hypothesis that specialists are under stronger selective constraints because they are pollinated by a single functional type of pollinator. The fact that such balancing selection is not detected for species with a mixed-pollination strategy suggests that selective constraints on a specific floral shape are perhaps less important, potentially because their various pollinators allow for a relatively efficient pollination across a wider shape space.

Another possibility that might explain the observed pattern of relaxed selection for species with a mixed-pollination strategy could be that this category is artificial and might instead consist of species with some degree of specialization to either hummingbirds or bats. If this was true, then a pattern of divergent selection within this category could be perceived as a less constrained evolution. While it is difficult to completely rule out this possibility, the distinct floral shape of species with mixed-pollination strategies and the fact that it evolved recurrently argues against such an hypothesis. Moreover, previous pollination studies in the group suggest that bats or hummingbirds always represent at least 20% of the visits of species with mixed-pollination strategies (Martén-Rodríguez et al., 2009).

It has been suggested that the presence of a constriction at the base of the corolla for Gesneriinae species with a mixed-pollination strategy could serve to present the nectar to the bats and guide hummingbirds for them to enter in contact with the reproductive organs (Martén-Rodríguez et al., 2009). This syndrome might thus represent a good candidate for floral shape evolution via trade-off effects, where the syndrome shape represents an adaptation to pollination by bats and hummingbirds, even though it might not be optimal for either of these (Aigner, 2001). Under this scenario, the constriction at the base of corollas with shapes otherwise adapted to bats (wide opening) might allow effective hummingbird pollination while maintaining bat pollination (Fig. 3). This strategy might be particularly successful where pollinators are scarce or vary through time, such as in island habitats. This hypothesis 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.

In light of this hypothesis, the rejection of the OU model for species with a mixed-pollination strategy might not indicate that there are no macro-evolutionary constraints on

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their flower shapes. Indeed, it is quite likely that the flower shape of zoophilous flowers are under some sort of balancing selection, and previous studies suggested that sexual floral traits show less variation than other floral traits (Cresswell, 1998). Instead, it might be that selective constraints are weaker for the species with mixed-pollination strategies than for more specialized strategies because of trade-off effects (Fig. 3) and that the small number of individuals with a mixed-pollination syndrome prevented a significant detection.

This study shows that distinct pollinator guilds can have different impacts on flower shape variation and evolution. In particular, it suggests that plants specialized on a single functional type of pollinators are likely to be under stronger evolutionary constraints. This study also helps to understand the apparent paradox between the observed evolutionary lability of pollination syndromes in angiosperms and the consistency of traits within pollination syndromes. Indeed, despite the substantial number of transitions (9) in a group of modest size (81 species), selection acts to stabilize corolla shapes over long periods of time for species with specialized syndromes. But, above all, these results reinforce Darwin's and Stebbins' views of the importance of pollinators in shaping floral diversity.

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Data availability

The data, R scripts and .xml files are all available as supplementary material.

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Table 1: Number of transitions between the different pollination syndromes according to the ancestral state reconstructions. The estimates are the values obtained on the maximum sum of clade credibility tree (Fig. 1). 95% credible intervals (in brackets) were obtained from the posterior distribution of trees. Ancestral state are in rows.

	bat	mixed-pollination	hummingbird
bat	0 [0, 4]	0 [0, 1]	0 [0, 2]
mixed-pollination	1 [0, 1]	11 [4, 18]	2 [0, 5]
hummingbird	2 [1, 3]	4 [0, 5]	38 [28, 42]

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Table 2: Evolutionary model selection and parameter values estimates. The models were fitted on 5000 species trees sampled from the posterior distribution. Median values and 95% credible intervals are reported. Positive ΔAIC values indicate support for the OU model and thus the presence of balancing selection. In cases where the 95% credible intervals (numbers in brackets) of the ΔAIC is below or includes 0, the parameters for the BM model are given instead of that of the OU.

PC1 and PC2 (multivariate)

Syndrome	ΔAIC	PC	σ	α	θ
mixed-pollination	-2.4 [-4.8, 0.58]	PC1	0.73 [0.53, 1.2]	–	–
		PC2	0.10 [0.067, 0.18]	–	–
hummingbird	14.9 [2.7, 66]	PC1	2.5 [1.2, 149]	242 [90, 18955]	0.16 [0.15, 0.17]
		PC2	11 [2.8, 827]	1675 [402, 1.2×10^5]	0.03 [0.03, 0.04]

PC1 (univariate)

Syndrome	ΔAIC	σ	α	θ
mixed-pollination	-0.28 [-1.2, 1.6]	0.72 [0.51, 1.3]	–	–
hummingbird	0.3 [-1.8, 6.0]	1.0 [0.74, 1.8]	–	–

PC2 (univariate)

Syndrome	ΔAIC	σ	α	θ
mixed-pollination	-2.0 [-2.0, -0.2]	0.08 [0.06, 0.12]	–	–
hummingbird	14.8 [3.4, 60.7]	14.1 [2.4, 2450]	1816 [297, 3.1×10^5]	0.035 [0.032, 0.039]

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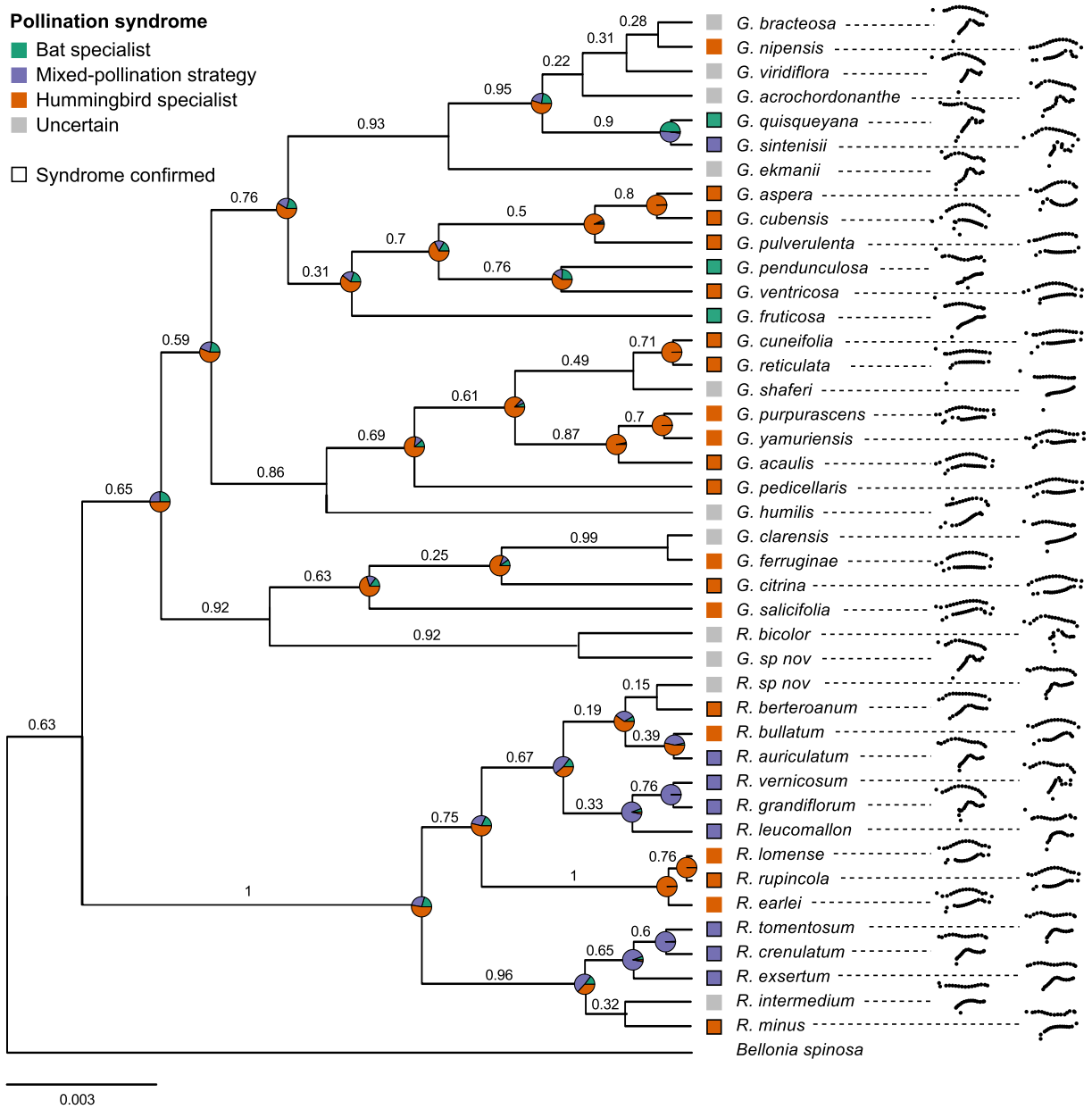

Figure 1: Species phylogeny showing mean corolla shapes (after Procrustes analysis). Pollinators are shown with those that have been confirmed indicated by a black contour. Pie charts represent the joint probability of each state at corresponding nodes as estimated by stochastic mapping; root estimates are not shown because they are unreliable Gascuel and Steel (2014). Clade posterior probabilities are shown above branches. Two outgroup taxa from tribes Gesnerieae and Trichosporeae are not shown.

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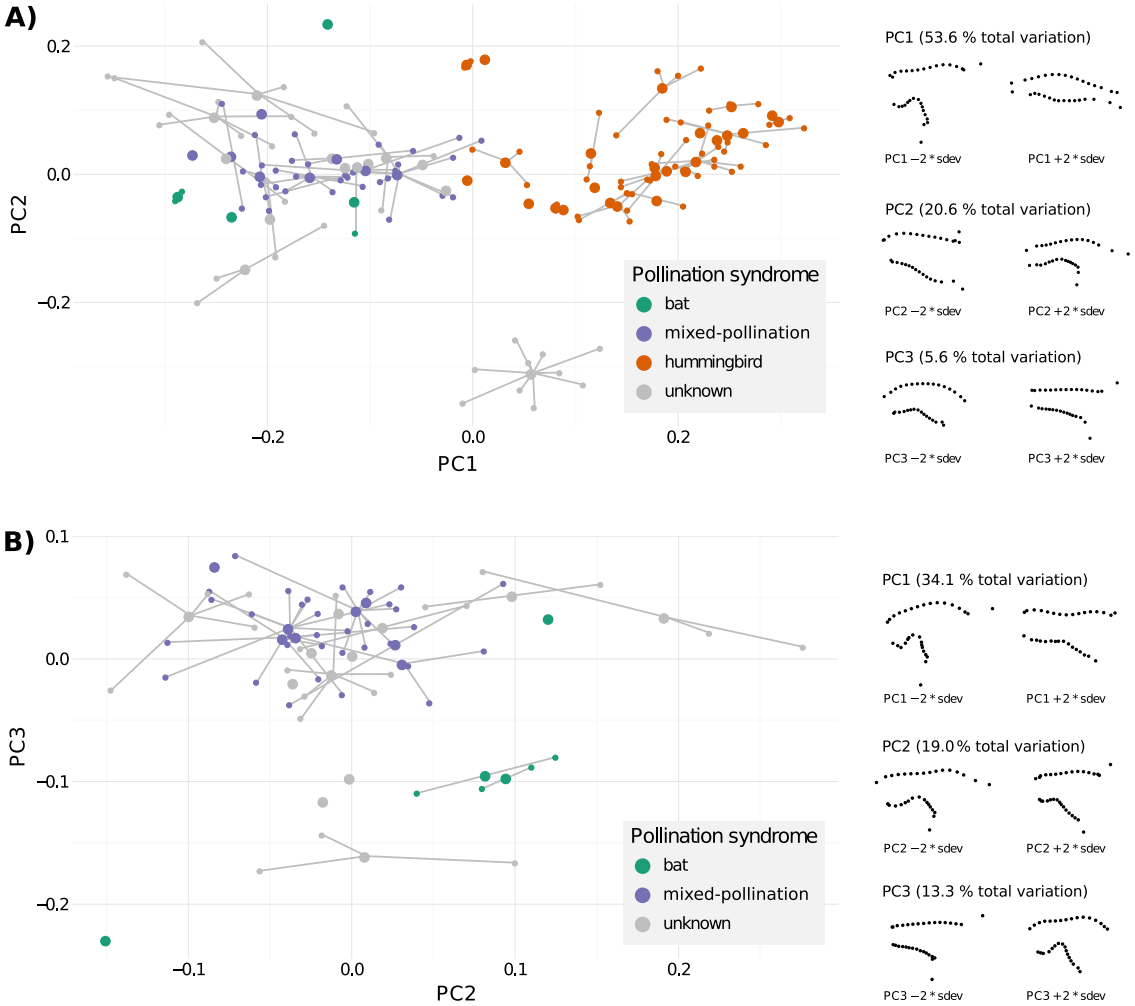

Figure 2: Principal component analyses showing the corolla shape morphospace for all species (A) and when excluding *G. humilis* and hummingbird pollinated species (B). The large dots on the plot represent the species means and the individual floral shapes (small dots) are associated with their species mean by gray lines. The corolla shape variation along the first three principal components are shown to the right (plus or minus 2 standard deviation from the mean shape).

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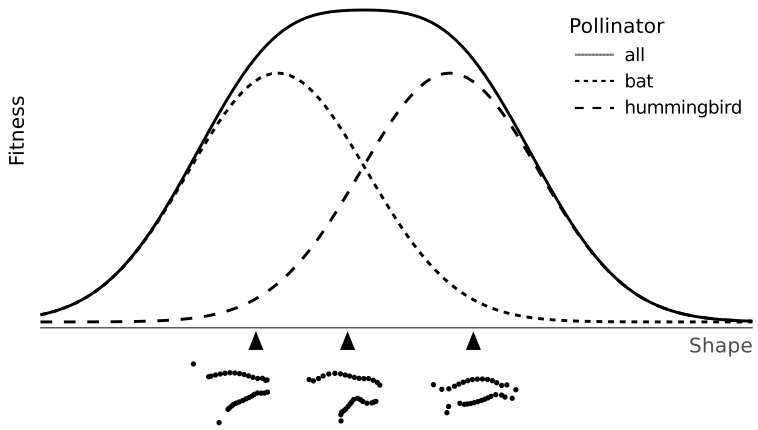

Figure 3: Imaginary example demonstrating how the generalist mixed-pollination syndrome might evolve via trade-off effects. The x-axis is a fictitious floral shape vector to illustrate the concept; real fitness landscapes are more likely to be multi-dimensional. Shapes to the left of the axis are better fitted to bat pollination and those to the right to hummingbird pollination. The curves represent the plant fitness for different flower shapes when bats, hummingbirds or both pollinators are present. Floral shapes of the mixed-pollination syndrome, with a constriction at the base of the corolla and a wide opening, have the capacity to be effectively pollinated by both types of pollinators even though their fitness is suboptimal with both bats and hummingbirds. Yet, this mixed-pollination shape could be favoured by selection if both pollinators are present.