

Evolution of intraspecific floral variation in a generalist–specialist pollination system

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Abstract

Intraspecific processes impact macroevolutionary patterns through individual variation, selection, and ecological specialization. According to the niche variation hypothesis, the broader ecological niche of generalist species results in an increased morphological variation among individuals either because they are constituted of diversified specialized individuals each exploiting a fraction of the species' niche or because they are constituted of true generalist individuals that experience relaxed selection. To test this hypothesis, we surveyed the individual floral morphology of species of Antillean Gesneriaceae, a group that has transitioned between specialization for hummingbird pollination and generalization multiple times throughout its evolutionary history. We characterized the profiles of corollas using geometric morphometrics and compared the intraspecific shape variance of specialists and generalists in a phylogenetic context. We used three approaches that differently accounted for the high dimensionality of morphological traits, the ancestral reconstruction of pollination syndromes over time, and the error associated with the estimation of the intraspecific variance. Our findings provide partial support for the niche variation hypothesis. If considering the whole shape in the analysis corroborated this idea, decomposing the shape into principal components indicated that not all aspects of the corolla exhibit the same pattern of variation. Specifically, pollination generalists tend to display greater intraspecific variation than specialists in terms of tubularity, but not of curvature. Accounting for the error in the variance estimation also reduced the support for the hypothesis, suggesting that larger sample sizes may be required to reach stronger conclusions. This study emphasizes the reciprocal influence between plants and their pollinators on floral morphology at different biodiversity scales and suggests that ecological strategies of species can affect patterns of morphological variation at macroevolutionary scales.

Keywords: plant pollination strategies, pollination generalists, pollination specialists, intraspecific variation, floral morphology, Antillean Gesneriaceae, macroevolution, niche variation hypothesis

Introduction

The patterns of morphological variation we observe at macroevolutionary scales have the potential to be influenced by processes occurring at the species level. For example, a higher degree of intraspecific trait variation may lead to a broader occupation of trait space at macroevolutionary scales. As a result, factors affecting intraspecific morphological variations, such as climate (Kuppler et al., 2020) or the ecological generalization of species (Van Valen, 1965), can shape macroevolutionary trends. Furthermore, intraspecific morphological variation contributes substantially to the overall trait diversity in communities (Siefert et al., 2015) and plays a crucial role across scales in driving their assembly and functioning (Bolnick et al., 2011; Violette et al., 2012; Westerband et al., 2021).

The impact of the level of ecological generalization on intraspecific variation has attracted much interest in the past.

Along the ecological specialist–generalist gradient, increased generalization is associated with a broader niche breadth, either in terms of resources (Grinnellian niche) or in terms of the functions a species performs in the ecosystem (Eltonian niche) (Devictor et al., 2010). A specialist thus exploits fewer resources than a generalist or contributes to a very specific function in the ecosystem. According to the ecological niche variation hypothesis (NVH), species with a broad ecological niche should have greater intraspecific morphological variation (Van Valen, 1965) (Figure 1A vs. B). This pattern can be caused by two distinct processes. On the one side, generalist species can be constituted of specialist individuals that occupy different ecological niches (Figure 1C). On the other hand, generalist species could be constituted of individuals that are intrinsically generalists, implying that they are all able to exploit a wide range of resources (Figure 1D).

Although there has been mixed support for the ecological NVH in the past (Grant & Price, 1981; Patterson, 1983),

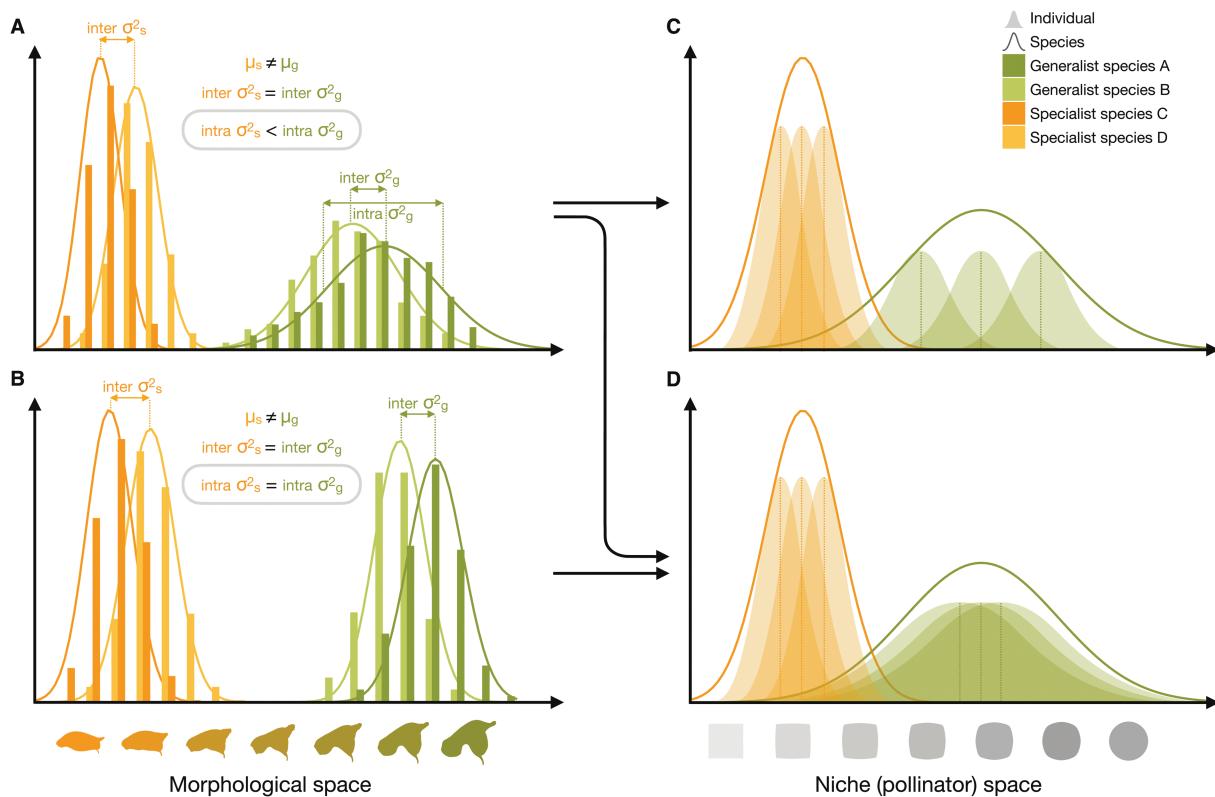


Figure 1. Illustration of the niche variation hypothesis (NVH) and three different ways (black arrows) that species can increase their ecological niche space (generalization). Interspecific morphological variations in Antillean Gesneriaceae are similar among pollination syndromes of hummingbird specialists (orange) and generalists (green) ($\text{inter } \sigma_s = \text{inter } \sigma_g$) even though they do not have the same mean floral shape ($\mu_s \neq \mu_g$). However, we do not know if the transition from specialist to generalist conforms to the NVH that predicts (A) higher intraspecific morphological variation for generalists than specialists ($\text{intra } \sigma_s > \text{intra } \sigma_g$) in contrast to (B) similar intraspecific morphological variation among pollination syndromes ($\text{intra } \sigma_s = \text{intra } \sigma_g$). On an ecological scale (niche space), generalization (niche breadth expansion) can occur (C) if generalist species are constituted of specialist individuals that occupy distinct niches or (D) if generalist species are constituted of intrinsic generalist individuals.

there is a tendency for generalists to display greater intraspecific morphological variation (Futuyma & Peterson, 1985). For instance, in hummingbird-pollinated plant species, long corolla tubes that are more ecologically specialized have lower intraspecific variation in corolla tube length (Fenster, 1991). However, there have been few tests of the NVH in a specific group by investigating simultaneously the intraspecific variation of multiple species while controlling for their evolutionary relationships. And despite the inherent importance of intraspecific variation across scales, few studies have incorporated it in macroevolutionary models.

In this study, we tested if differences in the degree of pollination generalization affect macroevolutionary patterns of intraspecific floral variation in a group of Antillean Gesneriaceae. In this group, pollination specialists are pollinated by a smaller subset of the potential pollinators in their environment (i.e., a single or a few species belonging to a functional group of pollinators) than pollination generalists (i.e., having more than one pollinator functional group). We hypothesize that floral traits of pollination generalists show greater intraspecific variation compared to specialists, as predicted by the NVH.

The Gesneriinae (Gesneriaceae) is a monophyletic group of ca. 80 species mostly endemic to the Antilles. In contrast to their relatives present on the American continent that are all pollination specialists (among those studied), several species

of the insular group are pollination generalists (Martén-Rodríguez et al., 2015). Indeed, although a large proportion of the species are hummingbird specialists and a few are bat or moth specialists, many species have a mixed-pollination generalist strategy being effectively pollinated by hummingbirds, bats, and insects (Martén-Rodríguez et al., 2009, 2010). Interestingly, the generalist pollination syndrome has evolved repeatedly in this group toward a convergent flower shape now found in all major islands of the Antilles (Joly et al., 2018; Martén-Rodríguez et al., 2010), making it an ideal group to study the impact of generalist pollination strategies on the macroevolutionary patterns of variation in the group.

Several studies have distinguished different pollination syndromes for these different reproductive strategies (Martén-Rodríguez & Fenster, 2008; Martén-Rodríguez et al., 2009, 2010, 2015). Species specialized for hummingbird pollination have a tubular corolla, a bright colour (red to yellow), and diurnal anthesis, and species specialized for bats have a campanulate (bell-shaped) corolla of pale colour (white to greenish) with nocturnal anthesis. Finally, species with a mixed pollination strategy have a campanulate corolla with a relatively large constriction at the base, of different colours and mottled patterns, with both diurnal and nocturnal anthesis (Joly et al., 2018; Martén-Rodríguez et al., 2009). One species of Gesneriinae also has a Heteroceran (moth) pollination syndrome with a yellowish tubular corolla.

A previous study of the group suggested that the flower shape of generalists is not an intermediate between the different specialist shapes (Joly et al., 2018) as might have been expected if the shape of generalists evolved under weak trade-offs (e.g., Aigner, 2001; Sahli & Conner, 2011). In contrast, the flower shapes of generalists occupy a completely distinct region of the floral morphospace, which was interpreted as their shapes being adapted specifically to be pollinated by distinct functional pollinators (Joly et al., 2018). At least at the interspecific level, the corolla shapes of generalist species did not show more variation than that of specialist species (Joly et al., 2018). However, we do not know if the corolla shape of generalists is more variable than that of specialists intraspecifically across the group, as would be predicted by the NVH, or if it mirrors the interspecific pattern of variation. In this research, we studied 261 individual flowers of 30 species to test whether generalists show greater intraspecific corolla shape variation. As there is currently no method capable of taking into account the complexity of multivariate geometric morphometric data, the stochastic nature of the evolutionary process, and measurement error, we used three approaches, each with its advantages and disadvantages, to test whether generalists have greater intraspecific variance in corolla shape than specialists in a clade of West Indian Gesneriaceae.

Materials and methods

Floral data

We quantified the corolla shape of species from the Gesneriinae subtribe using geometric morphometrics from flower photographs in strict lateral view. The floral image data set was compiled using the dataset used in Joly et al. (2018), to which we added new photographs mainly associated with herbarium vouchers or from the online database iNaturalist (available from <https://www.inaturalist.org>).

Pollinator information was obtained from field-based information (Faure & Joly, 2020; Martén-Rodríguez & Fenster, 2008; Martén-Rodríguez et al., 2009, 2010, 2015) or inferred from flower characteristics for species with unstudied pollination biology in the field as previous studies have shown that flower traits are a very good predictor of the pollination strategies in this clade (see Martén-Rodríguez et al., 2009).

Species names of West Indian Gesneriaceae were used in accordance with the Catalogue of Seed plants of the West Indies (Acevedo-Rodríguez & Strong, 2012) and Joly et al. (2023).

Phylogenetic reconstruction

The phylogeny of the Gesneriinae subtribe (Gesneriaceae) species studied was reconstructed for taking evolutionary relationships into account when comparing the floral shape variance of generalist and specialist species. DNA sequences of five nuclear genes (*CYCLOIDEA*, *CHI*, *UFR3GT*, *F3H*, and *GAPDH*) were collected from previous studies (Joly et al., 2018, 2023) to which sequences from 10 additional species were added to the dataset following the protocol of Joly et al. (2018). *Kohleria* sp. Regel “Trinidad” (Gesneriae) and *Codonoboea malayana* (Hook.f.) Kiew (Trichosporeae) were used as outgroups.

Sequences were aligned with MAFFT version 7 (Katoh & Standley, 2013), and the alignment was manually verified. A

species tree was reconstructed with *BEAST in BEAST version 1.8.4 (Heled & Drummond, 2010). Site substitution, molecular clock, and relative substitution rate were assigned independently to each gene. The site substitution model used was the GTR+Γ+I model (Yang, 1994a; Yang, 1994b) for each gene because over-parameterization of nucleotide substitution models brings very little bias compared to the under-parameterization of these models in Bayesian Markov Chain Monte Carlo (MCMC) analyses (Lemmon & Moriarty, 2004). An uncorrelated log-normal relaxed molecular clock was chosen for each gene. A birth-death prior was applied for the species tree, and population sizes were allowed to vary along the branches linearly with a constant population size at the root of the tree. Two MCMC chains were run for 50 million generations, with a recording of the parameter values every 2,000 generations, discarding the first 20 million generations as burn-in. The software Tracer (Rambaut et al., 2018) is used to verify the convergence of the chain for all parameters and the acquisition of good effective sample sizes (ESS). A maximum clade credibility (MCC) tree was then retrieved from the sampled trees.

Ancestral pollination regimes reconstruction

To test our hypothesis regarding the evolution of intraspecific variance, we used methods that require an explicit evolutionary scenario, specifically a phylogenetic tree with information about the character states of the species (specialist or generalist) along the branches of the tree. We used stochastic character mapping (Huelsenbeck et al., 2003) to simulate these evolutionary scenarios along the branches of the phylogeny. We first tested the fit of symmetrical and asymmetrical models of character evolution using an extended Mk model (Lewis, 2001) for discrete character evolution using the function `fitMk` from the R package `phytools` (version 1.0-1) (Revell, 2012). The model with the smallest Akaike information criterion (AIC) (Akaike, 1987) was then selected to perform 100 character simulations using the function `make.simmap` of the `phytools` package. The resulting ancestral trait maps were then used in the multivariate analysis of continuous trait evolution as well as the Bayesian analysis of joint inter- and intra-specific morphological evolution.

Characterization of corolla shape

To characterize the corolla shape of each individual floral profile, we used principles of geometric morphometrics. For each flower photograph, 32 landmarks were placed (6 landmarks and 26 semi-landmarks) (Joly et al., 2018) using the ImageJ software (version 1.5.3) (Schneider et al., 2012) and the Point-Picker plugin (<http://bigwww.epfl.ch/thevenaz/pointpicker/>). Two landmarks were positioned at the tips of the petal lobes, two at the base of the petal lobes, and two at the base of the corolla (see landmark placements in Supplementary Figure S1). The 26 semi-landmarks were evenly distributed along the upper and lower curvature of the corolla tube (13 points for each curve) between the base of the corolla and the base of the lobes to capture the shape of the corolla. We did not evaluate the variation of landmark placement between independent pictures because the error in data acquisition was estimated as minimal following this technique (i.e., 0.15% of the total variation was attributed to landmark placement Joly et al., 2018).

The specimens were aligned using a generalized Procrustes analysis (GPA) (Gower, 1975; Rohlf & Slice, 1990) using

the geomorph R package version 4.0.1 (Adams & Otárola-Castillo, 2013). The GPA translates all the shapes to the origin, scales them and rotates them to align the shapes as closely as possible. Landmark optimization during the Procrustes transformation was achieved by minimizing the curvature energy (Bookstein, 1997) rather than by minimizing the Procrustes distances (Rohlf, 2010), as recommended by Perez et al. (2006). For larger shape variations, as is the case with flower profiles of Gesneriaceae, the curvature energy minimization approach generally achieves better optimization of the displacement of the semi-landmarks and takes into account the local morphological deformation (Gunz & Mitteroecker, 2013; Perez et al., 2006). With curvature energy minimization, all landmarks and semi-landmarks are taken into account as well as the regularity of the analysed shape, while the points are taken independently with the Procrustes distance minimization method. This can make a semi-landmark slip beyond the limit of the curvature of the studied shape or exceed another semi-landmark (Gunz & Mitteroecker, 2013), which was observed with our data set. The coordinates of the aligned landmarks resulting from the Procrustes transformation (Kendall, 1984) were then used in the intraspecific floral shape variation analyses.

Floral morphospace

We used principal component analysis (PCA) to summarize the morphological variation in a few variables representing a maximum of the total variation of the corolla shape. Using the results of the Procrustean generalized analysis (the aligned sets of points coordinates), individual flower morphology was projected in a morphospace representing the phenotypical variation between individuals. The morphospace was obtained by PCA of the covariance matrix of landmarks using the function `prcomp` from the R package `stats` version 4.1.2. Diagnostic graphs and scree plot for the eigenvalues of the main PCA axes were generated using the R package `factoextra` (Kassambara & Mundt, 2020). To account for the morphological variation in the whole subtribe, we included the morphological data for all species with either a hummingbird specialist or a generalist syndrome in the morphospace that have at least three images per species, even if the species was not in the phylogenetic tree. Coordinates of individuals in the phylogeny were then selected in downstream comparative analyses.

Phylogenetic generalized least squares analysis of variance

To test whether generalist species exhibit greater intraspecific corolla shape variation (variance) than hummingbird-pollinated species while accounting for the phylogenetic non-independence of observations, we first used a phylogenetic generalised least squares (PGLS) approach (Grafen, 1989). We calculated the corolla shape variance using the standard formula $\frac{1}{n-1} \sum_{i=1}^n (x_i - \bar{x})^2$, where $(x_i - \bar{x})$ is the Procrustes (euclidean) distance calculated from the landmarks between the corolla shape of individual i and the mean shape of that species (\bar{x}). This approach has the advantage of considering the entire multivariate space in which the morphological shape of the species varies. The relationship between the intraspecific corolla shape variance and pollination strategy (specialists vs. generalist) was tested with a PGLS analysis using the function `g1m` from the R package `nlme` version 3.1-153 (Pinheiro et al., 2021). We allowed the

residuals of the model to be partly phylogenetically structured by using the `corPagel` correlation structure from the R package `ape` version 5.6-2 (Paradis & Schliep, 2019) and optimizing the lambda parameter. Estimating the strength of the phylogenetic structure in the residuals results in better statistical behaviour (Revell, 2010). Model fitting was done using the restricted maximum likelihood (REML), and the effect of the pollination strategy was tested using a *t*-test. The normality of the residuals and a plot of the residuals vs. fitted values were inspected to ensure the adequacy of the model.

Multivariate evolution of continuous traits evolution

To further test whether pollination specialists differ less in corolla shape variance than pollination generalists, we used Ornstein-Uhlenbeck (OU) evolutionary models that differ in whether specialists and generalists are allowed to have the same or different intraspecific variance and verified in the case of two distinct variances if generalists had greater corolla shape variation than specialists. Two models were fitted, one that used the corolla shape variance calculated from the Procrustes aligned floral shapes and previously used in the PGLS approach and another one using the variance estimated from the first three principal components (PCs) of the morphospace. In both cases, the natural logarithm of the variance (ln-variance) was used in the analyses.

We fitted univariate and multivariate OU evolutionary models with a single optimum (OU1) or different optima (OUM) of intraspecific variance for each pollination strategy (specialists and generalists) using the `mvOU` function of the R package `mvMROPH` (Clavel et al., 2015). A random root variance-covariance matrix was used, and the tree was scaled to unit length. These analyses were repeated on 100 stochastic mapping of the pollination strategies to consider the uncertainty associated with these reconstructions. To compare the model fit during each analysis, the Akaike Weights were calculated using the second-order information criterion (AICc) for each model and SIMMAP using the function `aicw` of the R package `mvMROPH`. The proportions of SIMMAPs supporting either the OU model with one optimum (OU1) or two optima (OUM) models of trait evolution were summarized.

Bayesian analysis of intra- and interspecific morphological evolution

General approach

To test whether both pollination strategies evolved towards distinct optimal values of intraspecific floral variation while taking into account the incertitude on the sample sizes of each species, we used a joint inter- and intraspecific variance evolution (JIVE) model integrated into a hierarchical Bayesian framework (Gaboriau et al., 2020; Kostikova et al., 2016). This approach takes as input the individual trait values for individuals in several species, a phylogeny with the pollination strategies mapped on the tree (specialist or generalist), and models of evolution for the intraspecific trait variance and for the mean trait. It then estimates the intraspecific variances and the parameters of the evolutionary models using MCMC. By using evolutionary models that allow different species to evolve under different regimes, it is possible to test, for instance, whether generalist species tend to evolve towards a greater intraspecific variance in corolla shape than specialist species (see Kostikova et al., 2016). This approach has the advantage of taking into account

the uncertainty in the intraspecific variance while modelling both the evolution of the intraspecific shape variance and mean shape across species. One drawback, however, is that it is an univariate approach and that the whole floral shape space cannot be taken into account in one analysis. We thus decomposed the global shape into PCs, and we analysed the first 3 principal axes of the floral morphospace with this approach. Only species present in the phylogeny and represented by at least three photographs were included in this analysis.

Bayesian analysis of phenotypic evolution

We determined a model of character evolution for mean corolla shape and intraspecific shape variance to use in the analysis. We used an OUM model for mean shape because this model was strongly supported in a previous study of corolla shape in West Indian Gesneriaceae (Joly et al., 2018). For the evolution of corolla shape variance, two models were considered. We fitted an OU1 model (OU process with one optimum) and an OUM model (OU process with one optimum per selective regime) that forces the specialists to have the same (OU1) or different (OUM) optimal intraspecific variances. We used the R package *bite* (Bayesian Integrative Models of Trait Evolution) version 0.4 (Gaboriau et al., 2020) to fit these models.

JIVE objects are generated as input for the implementation of the MCMC sampling for trait evolution models. JIVE objects were created for each simulation with the function *make.jive* of the *bite* package: the two models were estimated for each of the first three PCs for all 100 ancestral character histories produced by stochastic character mapping, resulting in a total of 600 JIVE objects. We used the default proposal functions with the following proposal parameters: mean variance (likelihood) per species (0.02), prior mean (α : 4, σ^2 : 3, θ : 0.2), and prior ln-variance (α : 4, σ^2 : 4, θ : 2). We used a uniform hyperprior for the ln-variance bounded between -10 and -1.

The MCMC chains for all 600 analyses were run for 4 million generations, with a sampling frequency of 200 generations, a burn-in phase of 10,000 generations, and 20 values of beta for the stepping-stone integration to allow for the estimation of the marginal likelihood for model selection (Xie et al., 2011) (see below). The sample size adjusted for auto-correlation (ESS) was estimated using the function *effectiveSize* from the R package *coda* version 0.19-4 (Plummer et al., 2006). Estimations plots (in particular the trace plots) were made using the functions *ggmcmc* and *ggs*

from the R package *ggmcmc* (Fernández-i Marín, 2016). The individual trace plots were verified for MCMC chains that stalled between temperature changes for the stepping stone integration; such analyses were repeated. The chains are then summarized per model and per principal component axes.

Model selection

To summarize the evidence provided by the data in favour of one statistical model over the other (OU or OUM), we used Bayes factors (Jeffreys, 1935; Kass & Raftery, 1995). The natural logarithms of the marginal likelihoods ($\ln(p(D|M))$) required for Bayes Factor calculation were calculated by stepping stone integration (Xie et al., 2011) using the function *marginal_liks* from the R package *bite*. The Bayes factor (BF_{10}) of model M_1 over the null (simpler) model M_0 is estimated as $BF_{10} = 2 \times (\ln(p(D|M_1)) - \ln(p(D|M_0)))$ and is interpreted following Kass & Raftery (1995); $BF_{10} > 2$ indicates positive evidence for M_1 , $BF_{10} > 6$ indicates strong evidence for M_1 , and $BF_{10} > 10$ indicates very strong evidence for M_1 . Here, model M_1 represents the model in which specialists and generalists have different variances.

Estimation of optimal variances for specialists and generalists

As another approach to test our hypothesis, we estimated the posterior probability that generalists have a greater intraspecific corolla shape variance than specialists ($\theta_g > \theta_s$) from the most complex model (mOUMvOUM). To calculate this posterior probability, we counted the number of generations from the post-burn-in chains with a temperature of 1 (no stepping stone integration) for which $\theta_g > \theta_s$. This was integrated over all stochastic mapping histories for each PC axis.

A summary of the approaches is present in Table 1 and a summarizing flowchart of the methods used is present in Supplementary Material (Supplementary Figure S2).

Results

Floral morphology

Images of 261 distinct individuals representing 30 Gesneriinae species (at least 3 individuals per species) with an identified or inferred pollination syndrome for hummingbird pollination and mixed-pollination were retrieved (see Supplementary Table S1). Pollination system data and sources of images are presented in Supplementary Tables S2 and S3, respectively.

Corolla morphospace explained 86.8% of the total shape variance in the first three principal components (PC) (see

Table 1. Details on the different approaches used in the intraspecific floral shape variation analyses.

| | PGLS | mvMORPH | JIVE |
|---|--------------------|---|------------------------------|
| Estimation of variance | On entire data set | On entire dataset or per principal component axis | Per principal component axis |
| Estimation of error (sample size) | No | No | Yes |
| Multivariate | Yes | Yes | No |
| Consideration of ancestral trait estimation (pollination regimes) | No | Yes | Yes |

JIVE = joint inter- and intraspecific variance evolution; PGLS = phylogenetic generalised least squares.

Figure 2 and [Supplementary Figure S3](#)); the other PC explained each less than 5% of the total variance. The first PC explained 64.4% of the total variance and characterized

tubular-shaped flowers from campanulate flowers with a wider opening and a median ventral constriction of the corolla ([Figure 2A and B](#)). The first axis is the main one discriminating

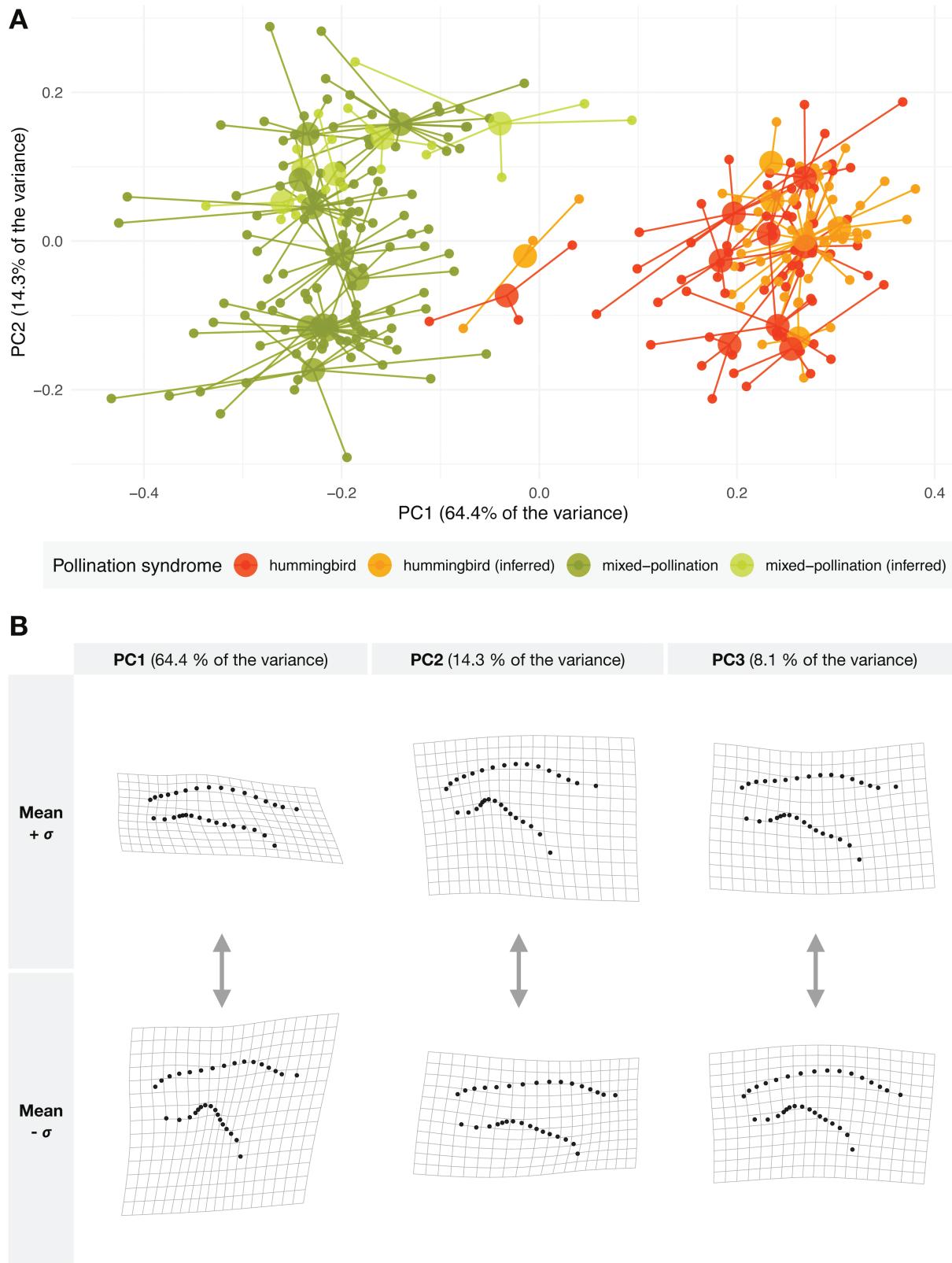


Figure 2. Corolla shape variation in Antillean Gesneriaceae. (A) Principal component analysis (PCA) of shape variation (morphospace). The arithmetic mean coordinate values for each species were calculated for each of the first three axes of the morphospace and are represented by the larger symbols, linked to individual values. (B) Table of the morphological variation along the first three axes of the morphospace.

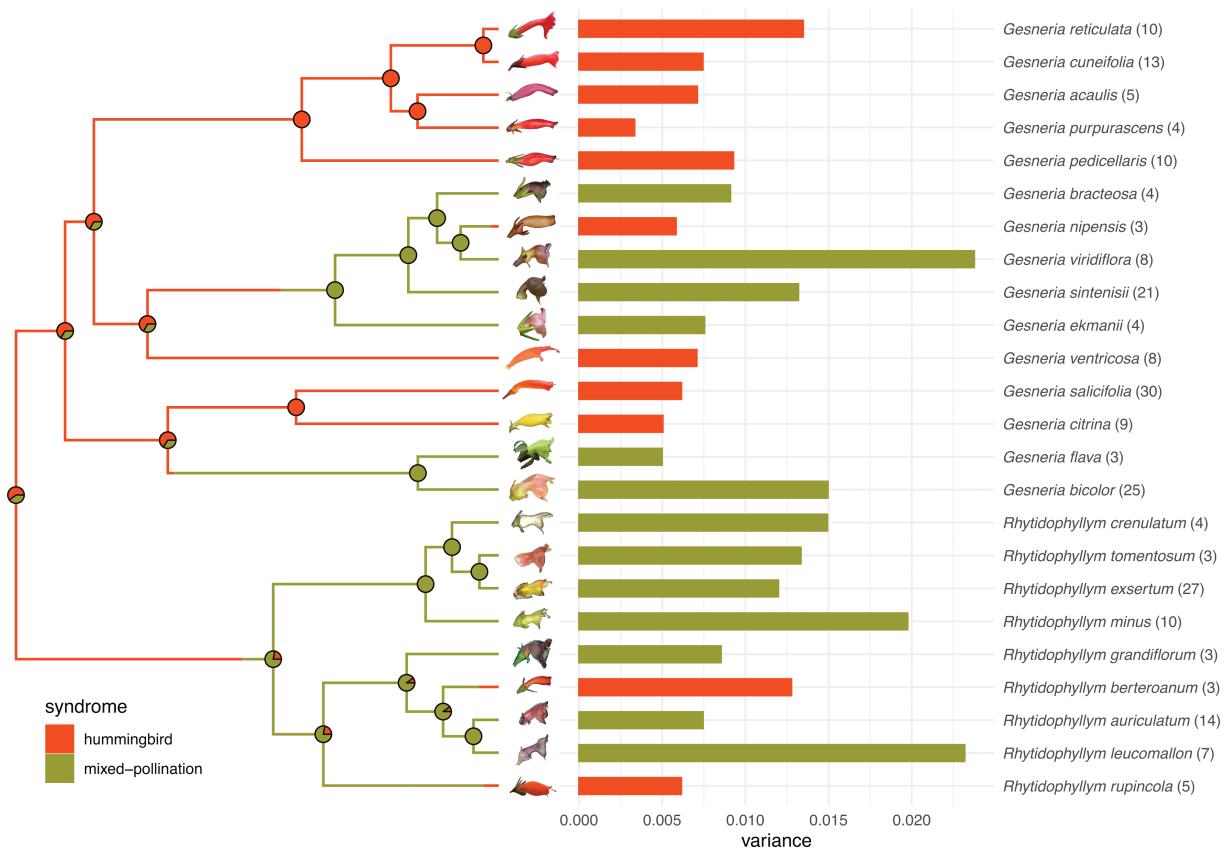


Figure 3. Phylogenetic tree and the total intraspecific corolla shape variance. The phylogenetic tree is a species tree (*BEAST) obtained from five nuclear genes. The pie charts at the nodes indicate the ancestral state probabilities of pollination strategies as estimated by stochastic mapping, and the colour on the branch represents one stochastic simulation outcome. Sample sizes for each species are indicated in parentheses. Photograph credit goes to John L. Clark, Abel Almarales-Castro, Silvana Martén-Rodríguez, François Lambert, and Simon Joly.

between specialist and generalist species. The second PC axis accounted for 14.3% of the total variance and broadly characterizes a change in corolla curvature, with more curved flowers showing more pronounced corolla constriction. The third PC axis accounted for 8.1% of the total variance and characterized a smaller difference in the shape of the corolla curvature. These floral variations along the axes are concordant with Joly et al. (2018).

Phylogenetic reconstruction

We gathered sequence data from most of the five selected genes for 110 individuals representing 53 species of the Gesneriinae tribe (Supplementary Table S4). In total, 23 individuals representing 15 species, including 10 additional species since the phylogeny presented in Joly et al. (2018), were added to the phylogenetic reconstruction of the subtribe Gesneriinae. The genus *Bellonia* L. is sister to the rest of the subtribe with branch support of $pp = 0.99$. *Rhytidophyllum* Mart. and *Gesneria* L. formed distinct groups, with less support for the *Gesneria* genus (1.00 and 0.59, respectively). In comparison with the phylogeny from Joly et al. (2018), *Gesneria depressa* (Griseb.) Urb. and *Gesneria pulverulenta* Alain were not included in the phylogenetic analysis because they importantly decreased the tree resolution. Only two genes (GCYC and F3H) were sequenced for *G. depressa* and only one (GCYC) for *G. pulverulenta*, from one individual each. This phylogeny is overall congruent with the one from Joly et al. (2018). For the following analyses, the species tree

(Supplementary Figure S4) was pruned to remove all but the 24 hummingbird specialists and generalist species that had at least three profile images (11 specialists and 13 generalists) (Figure 3).

Ancestral character mapping of syndromes

For the ancestral reconstruction of pollination strategies, the equal rate (ER) model ($AIC = 68.03$) was selected over the asymmetrical rate (SYM) model ($AIC = 70.03$). Stochastic simulations on 100 trees suggest six changes between generalist and specialist strategies on average. The estimated number of transitions between states is higher from generalist to hummingbird specialists (3.75) than from hummingbird specialists to generalists (2.25). Hummingbird specialist was the most likely ancestral state for the ancestor of *Gesneria* and *Rhytidophyllum* with a probability of 0.6 (Figure 3). The proportion of time spent in the hummingbird specialist state is also greater than the proportion of time spent in the generalist state (51.7% and 48.3%, respectively).

Phylogenetic generalized linear model

The species intraspecific variances of corolla shape (Figure 3) estimated from the Procrustes aligned corollas were used in a phylogenetic generalized linear regression to test whether pollination generalists have a greater intraspecific corolla shape variance. Distribution of the normalized residuals and fitted values is available in Supplementary Material (Supplementary Figure S5). The results showed that pollination strategy significantly influences floral shape variation ($p = 0.0064$; Table 2).

The estimated variance of specialists was 0.0093, and generalists had a variance 0.0069, greater than that of specialists (**Table 2**). The strength of the phylogenetic signal of the residuals ($\lambda = -0.0266$) suggests that closely related species have more different intraspecific variances than would be expected under a neutral (i.e., Brownian) model of evolution.

Multivariate models of continuous traits evolution

OU models with one optimum (OU1) or two optima (OUM) of trait variance were fitted on the ln-variance of the first three PCs of the morphospace, the first three PCs combined, and on the Procrustes variance estimated from the whole dataset. The OUM model was supported in the analysis of PC1 for the totality of the simulated maps of ancestral syndromes (AIC weights of the OUM model > 0.50; **Figure 4A**). On the contrary, the model OU1 was the model with the best fit for the totality of the simulated maps when considering independently PC2 and PC3, as well as the three PCs analysed simultaneously in the multivariate approach (AIC weights of the OUM model < 0.50; **Figure 4A**).

The OUM model is selected for PC1 (mean = 0.577, SD = 0.0281) and even more strongly for the variances calculated from aligned Procrustes shapes (mean = 0.9241, SD = 0.01923699) (see **Figure 4A**). On the other hand, the OU1 model is increasingly selected for PC2 (mean = 0.405, SD = 0.0146), PC3 (mean = 0.222, SD = 0.0147), and the joint PC1+2+3 (mean = 0.105, SD = 0.0367). Even though the first PC represents 64.5% of the total variation, the modelization of the trait variance along the first PC alone reveals the opposite model selection compared with the first three components taken into account simultaneously (**Figure 4A**). The OU1 model best fitted the first three PCs taken simultaneously

PC1+2+3, even more so than when the PC2 and PC3 were analysed independently.

Considering the OUM model, variance estimates (θ) revealed concordant values of variance for specialists (θ_s) and generalists (θ_g) across analyses as values of variances along the PC axes whether analysed independently (PC1, PC2, PC3) or jointly (PC1+2+3) are clustered together (**Figure 4B**). The θ_s are lower than θ_g for the PC1, and PC3 (with the exception of four SIMMAPs for PC3) and θ_s are higher than θ_g along the PC2 (**Figure 4B**). The multivariate approach that considers the variance calculated from the Procrustes aligned floral shapes however places the totality of the estimations using the 100 SIMMAPs above the $\theta_s = \theta_g$ limit (**Figure 4B**). When using the overall shape variance as in the PGLS approach, the θ_s is also estimated lower than θ_g (**Figure 4C**).

Bayesian analysis of intra- and interspecific morphological evolution

We also used JIVE to compare OU1 and OUM models of evolution by modelling both intra- and interspecific trait evolution. Bayes factors were calculated for each of the first three axes of the floral morphospace and for 100 simulated evolutionary scenarios of pollination strategies to account for the uncertainty in the ancestral states along the branches of the phylogeny. This resulted in 100 different BF values for each axis of the morphospace.

For each principal component (PC) of the morphospace, we found great variation in BF values, ranging from highly negative (suggesting a rejection of the model that specialists and generalists have different variances) to highly positive (selection of the most complex model) (**Figure 5**). Complete BF details for each SIMMAP are available in Supplementary Material (**Supplementary Table S5**). No distinct trend of evidence for one or the other model was found overall; each PC had character simulations that supported the simpler model, the more complex one, or none of them, and PC1 and PC3 had sensibly the same amount of SIMMAPs in all categories (**Table 3**). In contrast, PC2 tended to support the simpler model (mOUMvOU) overall with a single optimal variance value in terms of corolla shape variance.

For the first axis, even though it represents a smaller proportion of the SIMMAPs, the OU1 model is supported by

Table 2. Phylogenetic generalized least squares model results ($n = 24$ species) for the effect of pollination syndrome on the whole corolla shape variation.

| Coefficients | Value | SE | T-value | p-value |
|---------------------|--------|--------|---------|---------|
| Intercept | 0.0093 | 0.0017 | 5.6091 | 0.0000 |
| Generalist syndrome | 0.0069 | 0.0023 | 3.0123 | 0.0064 |

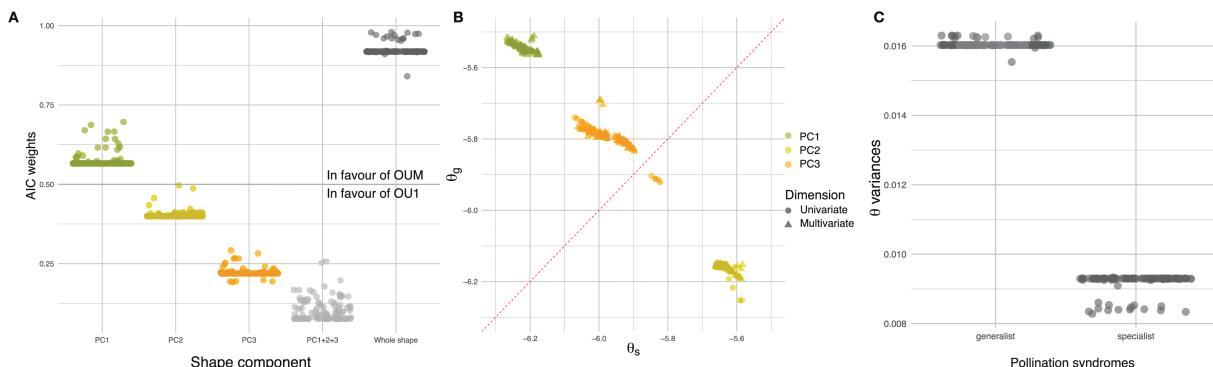


Figure 4. Results of the Ornstein–Uhlenbeck (OU) model selection and parameter estimation with mvMORPH. (A) akaike information criterion (AIC) weights of the multivariate OU model of evolution using the first three components of the floral morphospace independently and jointly, using their calculated intraspecific variation on the total Procrustes aligned shapes. (B) Optimal values of variances (θ) estimated for generalists and specialists using their calculated intraspecific variation on the total Procrustes aligned shapes in the multivariate approach mvMORPH. (C) Optimal values of variances (θ) estimated for generalists and specialists using their calculated intraspecific variation on the total Procrustes aligned shapes in the multivariate approach mvMORPH. Each point represents one character simulation (SIMMAP).

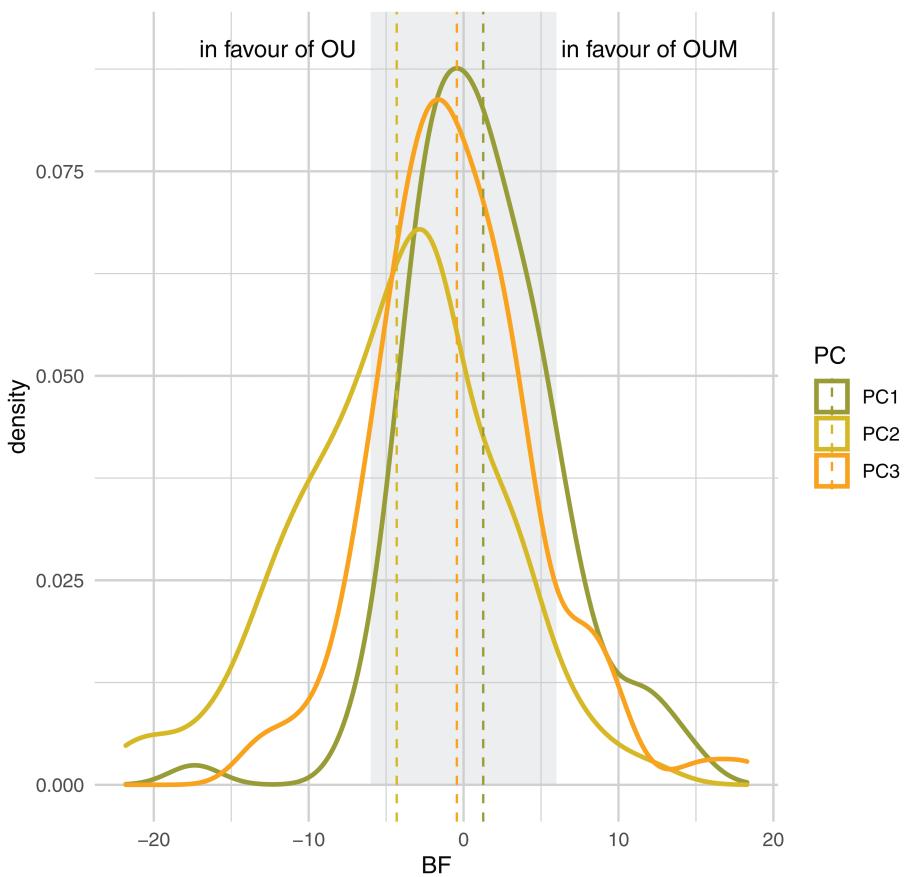


Figure 5. Density of Bayes factor (BF) values for each principal component (PC) of the morphospace. The arithmetic mean of the BF for each PC is represented by a dashed vertical line. The BF confidence interval [-6, 6] is indicated by a grey-shaded area. $BF = 2 \times (\text{marginal likelihood } m_{\text{OUM}}/m_{\text{OU}} - \text{marginal likelihood } m_{\text{OUM}}/m_{\text{OU}})$.

Table 3. Number of SIMMAPs of 100 following each Bayes factor (BF) category for each of the three first principal components of the morphospace: Evidence in favour of OU1 ($BF < -6$), no evidence for either model ($-6 < BF < 6$), and evidence in favour of OUM ($6 < BF$).

| Principal axis of the morphospace | $BF < -6$ | $-6 < BF < 6$ | $6 < BF$ |
|-----------------------------------|-----------|---------------|----------|
| PC1 | 2 | 84 | 14 |
| PC2 | 35 | 58 | 4 |
| PC3 | 12 | 76 | 10 |

ancestral character maps that display a higher probability of the generalist syndrome at the root of the trees (upper left panel in Figure 6). On the contrary, the OUM model is supported by ancestral character maps that have a higher probability of a specialist at the base of the tree (bottom left panel in Figure 6). A more recent apparition of the generalist syndrome suggests a multivariate model (OUM), whereas longer time spent as generalists suggests the simpler single optimum model (OU1). A clear pattern for PC2 and PC3 (two last columns in Figure 6) is however more difficult to distinguish.

Because the use and accuracy of model selection in Bayesian phylogenetic modelling have been debated (Lartillot, 2022), especially when none of the competing hypotheses may be true, it may be a good approach to use other evidence to test biological hypotheses. Here, we estimated the posterior probability that generalists have a higher intraspecific variance than specialists from the MCMC analysis in which the

optimal variance for generalists and specialists was estimated ($m_{\text{OUM}}/m_{\text{OU}}$).

The probability that generalists have a higher intraspecific corolla shape variance than specialists $p(\theta_g > \theta_s)$ was 0.95 for PC1, 0.32 for PC2, and 0.92 for PC3 (Figure 7). The intraspecific variance for generalists was estimated to be 2.01 times that of specialists for PC1, 0.82 for PC2, and 1.49 for PC3. The summary of the estimated model parameters is provided in Supplementary Table S6, and the ESS of the parameters for the combined MCMC analyses are provided in Supplementary Table S7.

Discussion

According to the NVH, a greater ecological niche is expected to lead to greater intraspecific morphological variation (Van Valen, 1965). We, therefore, hypothesized that pollination generalists should vary more in floral shape than specialists at the intraspecific level (Figure 1A vs. B). Using floral morphologies of Antillean Gesneriaceae, we tested if generalists have greater intraspecific corolla shape variance than specialists.

We used three distinct approaches to test this hypothesis, each of which has different advantages and disadvantages. The simpler approach aimed at comparing the intraspecific variation in corolla shape among species while correcting for the phylogenetic correlations in the data using a phylogenetic generalized least square approach (PGLS). The PGLS

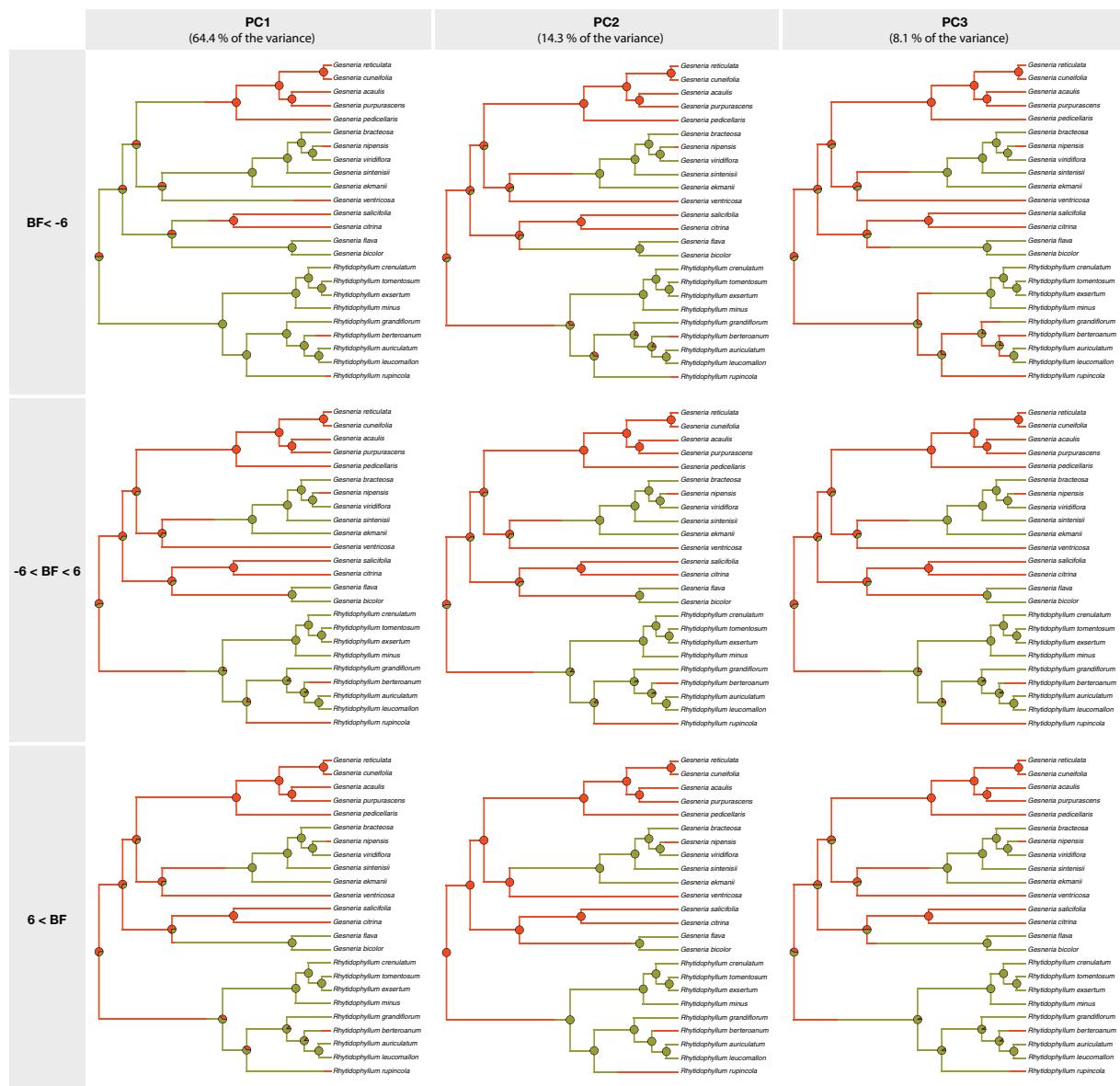


Figure 6. Summary of the simulated maps of ancestral syndromes (SIMMAPs) for specialists for hummingbird pollination (orange) and generalists (green) using the first principal components of the morphospace (PCA) and for the three Bayes factor (BF) categories: $\text{BF} < -6$ (in favour of OU), $-6 < \text{BF} < 6$ (no model favoured), and $6 < \text{BF}$ (in favour of OUM).

approach considers the whole corolla shape but does not consider the error in the estimation of the variance and does not use explicit models of pollination strategy evolution. The other two approaches tested the hypothesis by comparing two OU stochastic models of evolution with explicit models of pollination strategy evolution, one with a single joint optimal value of trait variation for both pollination strategies (OU1) and one another with two distinct optima of trait variation for each syndrome, respectively (OUM). The second approach (mvMORPH) takes into account the multi-dimensionality of the data but did not take into account the error involved in the estimation of the variance of the species. Lastly, the hierarchical Bayesian approach (JIVE) allowed accounting for the error in estimating species variances when estimating the optimal variances for the specialist and the generalists, but it cannot consider the whole corolla shape, which had to be simplified to PCs of variation.

Because inconclusive results were obtained with some of the different approaches, a strong conclusion could not be reached. Nevertheless, some trends are distinguishable. The PGLS and mvMORPH approaches support higher intraspecific corolla shape variation for generalists than specialists when the whole floral shape is considered. This was also the case for the first PC of the morphospace when analysed with mvMORPH, which describes floral tubularity (64% of the total corolla shape variation). The JIVE approach also points toward increased variation for generalists for PC1 when the parameters of the OUM model are considered, but this more complex model is not supported statistically. In contrast to when the global shape or PC1 are considered, there is no support for greater variation in the corolla shape for generalists along the second PC. Although the variation along PC3 points towards greater variation in generalists (multivariate mvMORPH), the result is not significant when

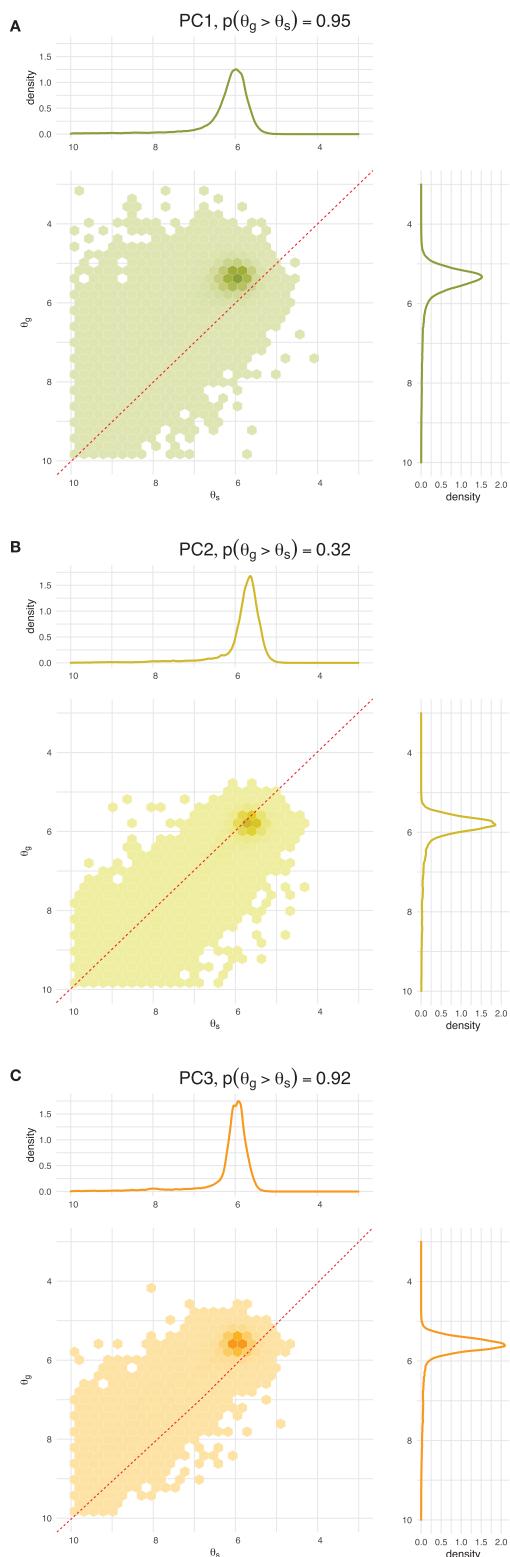


Figure 7. Distribution of the joint estimation of the optimal floral variance of specialists (θ_s) and generalists (θ_g) for all SIMMAPS. The densities of θ_s and θ_g are represented for the first three principal components PC1 (A), PC2 (B), and PC3 (C) and along both axes (top and left). The red dashed line indicates the limit of $\theta_s = \theta_g$.

analysed alone. This highlights that different axes of variation do not necessarily follow the global pattern of floral variance evolution.

A few things might explain the differences in results between the methods. First, there is more support for a greater variance for generalists when the whole corolla shape is considered compared to when the shape is reduced to PCs, even when the first three PCs are analysed simultaneously. In this latter case, the lack of statistical support for the OUM model could be due to a lack of power because of the additional parameters that must be included in the multivariate model. Because JIVE does not allow us to analyse the whole corolla shape, the lack of statistical support with this approach could be partly due to this decomposition of shape. This said, we also observed that for the analysis of individual PCs, there was less support for the OUM model for JIVE than for mvMORPH. This is likely because the JIVE approach takes into account the uncertainty in the estimation of the intraspecific variance, which other methods do not. Because our sample sizes are relatively small, the lack of statistical significance with JIVE might also point to low power. Ideally, future development of the JIVE model should include the possibility of assessing the variance from multidimensional datasets. This is even more relevant because it is known that analysis of only the first few principal axes of a multivariate dataset as independent traits with evolutionary models can lead to erroneous conclusions (Revell, 2009; Uyeda et al., 2015), which prompted the development of frameworks of high-dimensional phylogenetic comparative methods (Clavel et al., 2018).

It is interesting to note that character history simulations strongly impacted the JIVE results because some SIMMAPs supported one model and others rejected it for the same principal component of shape variation. For instance, the longer the generalist syndrome took to arise within Antillean Gesneriaceae, the higher their intraspecific morphological variation seems to be in comparison with specialists. Therefore, the lack of certainty in the ancestral evolution of the pollination strategies seems to affect the JIVE results and highlights the importance of considering this source of uncertainty in such evolutionary analysis because the reliance on a single SIMMAP could have completely biased our conclusions. Nevertheless, the impact of the SIMMAPs was much more minor for the mvMORPH analysis, although it affected the estimates of the parameter values and the support for the alternative models.

Although not conclusive, our results point towards the idea that generalist species have greater intraspecific morphological variation. The NVH was tested here using a group of closely related species that consist of several independent origins of a generalist strategy, and we measured morphological variation on a key ecological trait: the corolla (Martén-Rodríguez et al., 2009). These results have interesting implications for this group. Indeed, because we know that variation among species in corolla shape is similar for specialists and generalists (see Joly et al., 2018), the greater intraspecific variation of generalists suggests a more complete filling of the corolla morphospace for generalists compared to specialists and/or a greater overlap in the corolla shape variation among generalist species. This illustrates how ecological strategies of species could affect macroevolutionary patterns of morphological variation.

Van Valen (1965) underlined that greater intraspecific morphological variation for generalists can occur either if generalists are constituted of specialist individuals (Figure 1c) or if the generalists are constituted of intrinsically generalist

individuals (Figure 1D). Although we did not directly measure the niche of individuals in this study, previous pollination studies suggest that individuals of generalist species are all pollinated by a variety of pollinators (e.g., Martén-Rodríguez et al., 2009, 2015). This suggests that in Antillean Gesneriaceae, the greater intraspecific corolla shape variation of generalists is due to more relaxed selective constraints than specialists (Rosas-Guerrero et al., 2011). One scenario that may lead to this pattern is if, for instance, different populations of generalist plants are pollinated by the same functional group of pollinators (e.g., hummingbirds, bats and bees), but in different proportions due to the environment (e.g., altitude). This could exert different selection pressures on the corolla in different populations, something not possible for pollination specialists pollinated by a single species.

The evolution of the generalist pollination strategy in the studied group occurs in an insular context. Islands are generally pollinator depauperate environments compared to the mainland (MacArthur & Wilson, 1967) where pollination specialists are more vulnerable to pollination inefficiency or failure (Armbruster, 2017). Moreover, temporal variation in pollinator communities, which may result from frequent storms and hurricanes in the Caribbean islands, may further favour the evolution of pollination generalists. Indeed, temporal variation offers fewer opportunities for specialist refuges, increasing their risk of extinction (Johnson, 2010). In Antillean Gesneriaceae, floral visitation rates are lower and pollen limitation of fruit set is greater in species with specialized compared to generalized pollination systems (Martén-Rodríguez and Fenster, 2010; Martén-Rodríguez et al., 2015). The generalist strategy, which evolved independently several times in Antillean Gesneriaceae (Martén-Rodríguez et al., 2010; Joly et al., 2018), can thus be considered as an alternative mechanism to assure their reproduction. At the same time, the paucity of pollinators can also reinforce specialization as a result of a lack of alternative pollinators (Armbruster, 2017). In this context, the evolution of autonomous self-pollination could have allowed the maintenance of hummingbird specialists in Caribbean Gesneriaceae (Martén-Rodríguez et al., 2010). In addition, higher levels of self-pollination could reduce genetic variation, which could reduce floral trait variation and partly explain the results we observed.

The hummingbird specialist syndrome is not the only specialist strategy within the genera *Gesneria* and *Rhytidophyllum*. There are known moth and bat specialists in the group (Martén-Rodríguez et al., 2010), although there are very few species that fall in these categories (only *G. humilis* L. is specialized for moth pollination, and *G. quisqueyana* Alain, *G. pedunculosa* (DC.) Fritsch, *G. fruticosa* (L.) Kuntze, *G. shaferi* Urb. subsp. *depressa* (Griseb.) L.E. Skog and *G. claren-sis* Britton & P. Wilson are bat specialists). Most of these species are narrowly distributed and occur in remote populations; hence, they could not be included in this study. Nevertheless, it would be interesting to include them in a future study to compare their corolla shape variation with those of hummingbird specialists and generalists pollinated by bats and hummingbirds.

In conclusion, our study sheds light on the evolutionary patterns of intraspecific variation in corolla shape in Antillean Gesneriaceae, revealing the crucial role of pollination strategies in shaping their floral morphology, both at the micro- and macroevolutionary levels. Although our findings are not

conclusive, they suggest that pollination generalists tend to exhibit greater intraspecific variation in corolla shape than pollination specialists, particularly in terms of general shape and tubularity, while they show similar variation in terms of curvature. These results not only support the NVH but also show that not all aspects of the corolla show the same pattern of variation. This increased variation in generalists is likely to be due to relaxed selective constraints on generalists that can be pollinated by various functional types of pollinators and has implications on the macroevolutionary pattern of variation in the group. As the Caribbean environment is rapidly changing due to anthropogenic and climatic impacts, understanding the impact of pollination strategies on floral diversity and evolution in this region is a timely and critical topic.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

Scripts and data are available on Dryad (<https://doi.org/10.5061/dryad.hqbzh1nh>).

Authors contributions

Marion Leménager (Conceptualization [Equal], Data curation [Supporting], Formal analysis [Lead], Methodology [Equal], Visualization [Lead], Writing—original draft [Lead], Writing—review & editing [Equal]), John Clark (Data curation [Equal], Writing—review & editing [Supporting]), Silvana Martén-Rodríguez (Data curation [Equal], Writing—review & editing [Supporting]), Abel Almarales-Castro (Data curation [Supporting], Writing—review & editing [Supporting]), and Simon Joly (Conceptualization [Equal], Funding acquisition [Lead], Methodology [Equal], Supervision [Lead], Writing—original draft [Supporting], Writing—review & editing [Equal])

Funding

This work has been financially supported by the Montreal Botanical Garden and a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to S.J. (No. 05027-2018), (Dirección General de Personal Académico grant No. PAPIIT-IN224920) to S.M.R., and a Research and Exploration Grant from the National Geographic Society to J.L.C. (No. 9522-14).

Acknowledgments

The authors thank the Advanced Research Computing platform provided by the Digital Research Alliance of Canada and Geneviève Lajoie for computing resources. We thank Théo Gaboriau for constructive methodological discussions and Geneviève Lajoie for enriching discussions on intraspecific trait variation. The authors also thank those who provided images: iNaturalist platform and users as cited in SI, François Lambert, Thomas E. Talpey, Étienne Léveillé-Bourret, and the United States Botany Research Greenhouses (USBRG).

Conflicts of interest

The authors have no conflict of interest to declare.

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