

Evolutionary breakdown of pollination specialization in a Caribbean plant radiation

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Summary

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Received: 6 March 2010
Accepted: 4 May 2010

New Phytologist (2010) **188**: 403–417
doi: 10.1111/j.1469-8137.2010.03330.x

Key words: Antilles, autogamy, floral traits, *G-CYCLOIDEA* (*GCYC*), Gesneriaceae, internal transcribed spacer (ITS), islands, phylogeny, pollination systems.

- Ecological generalization is postulated to be the rule in plant–pollinator interactions; however, the evolution of generalized flowers from specialized ancestors has rarely been demonstrated. This study examines the evolution of pollination and breeding systems in the tribe Gesnerieae (Gesneriaceae), an Antillean plant radiation that includes specialized and generalized species.
- Phylogenetic reconstruction was based on two nDNA markers (internal transcribed spacer (ITS) and *G-CYCLOIDEA* (*GCYC*) and morphology. The total evidence Bayesian phylogeny was used for assessment of floral character evolution using Bayesian stochastic character mapping.
- Mapping of the pollination system resulted in at least two origins of bat pollination and two origins of generalized pollination (bats, moths and hummingbirds). The evolution of bat pollination was associated with floral transitions reflecting the chiropterophilous floral syndrome. The evolution of generalization was associated with subcampanulate corollas. Autonomous breeding systems evolved only in hummingbird-pollinated lineages.
- The correlated evolution of floral traits and pollination systems provides support for the pollination syndrome concept. Floral transitions may have been favored by the low frequency of hummingbird visitation in the Antilles, while the presence of autonomous pollination may have allowed the diversification of ornithophilous lineages. Results suggest that pollinator depauperate faunas on islands select for the evolution of reproductive assurance mechanisms, including generalization and autogamy.

Introduction

Animal pollinators have played a crucial role in the diversification of angiosperm flowers (Grant & Grant, 1965; Stebbins, 1970; Fenster *et al.*, 2004). Evidence for the importance of pollinator-mediated selection comes from a large number of studies across the fields of evolutionary ecology (Pauw, 2006; Reynolds *et al.*, 2010), and comparative biology (Armbruster, 1988; Kay *et al.*, 2005; Smith *et al.*, 2008). However, our understanding of how plant–pollinator interactions have influenced floral diversification is still incomplete. For example, the level of specialization in plant–pollinator interactions is not known for most

plant species, and its influence on floral evolution is a current subject of discussion (Waser *et al.*, 1996; Johnson & Steiner, 2000; Fenster *et al.*, 2004; Ollerton *et al.*, 2007). The often-cited association between floral diversification and increased specialization in pollination systems is frequently rejected in phylogenetically informed studies (Kay & Sargent, 2009; Knapp, 2010). In fact, if we consider specialization at the level of functional groups of pollinators (rather than number of pollinator species), pollination–system transitions have occurred in all directions: from generalized to specialized (Chase & Hills, 1992), between specialized systems (Armbruster, 1988; Kay *et al.*, 2005; Wilson *et al.*, 2007; Whittall & Hodges, 2007) and

from specialized to generalized systems (Armbruster & Baldwin, 1998). However, there is limited evidence to draw solid inferences about the frequency of these different evolutionary pathways and their importance in floral diversification.

Oceanic archipelagos provide a great opportunity to study the evolution of specialization and generalization in plant–pollinator interactions. Similar pollinator faunas on different islands may generate parallel selective environments resulting in the convergent evolution of floral phenotypes. Furthermore, depauperate island pollinator faunas are expected to impose selective pressures on plant pollination and breeding systems to increase reproductive output by shifting to alternative modes of pollination. When the original pollinators are absent or scarce, selection should favor the evolution of generalized pollination systems, wind pollination, and autonomous breeding systems (Barrett, 1996). However, while unspecialized flowers prevail in remote oceanic islands, some plant groups exhibit elaborate floral phenotypes (Antillean Gesneriads; Skog, 1976; Hawaiian Lobeliads, Givnish *et al.*, 1995; Hawaiian mints, Lindqvist & Albert, 2002). The presence of morphologically specialized flowers may reflect the occurrence of novel or more effective pollinators. Alternatively, the maintenance and diversification of specialized lineages could be favored by the presence of breeding systems that provide reproductive assurance in insular environments (Jain, 1976; Fenster & Martén-Rodríguez, 2007).

Although a considerable number of plant phylogenies are available for island plants, our understanding of the selective forces underlying pollination system evolution is limited by the number of pollination ecology studies available for medium- to large-sized plant taxonomic groups. For example, the Caribbean islands, considered one of the biodiversity hotspots of the planet (Myers *et al.*, 2000), provide numerous examples of plant radiations (Skog, 1976; McDowell & Bremer, 1998; Santiago-Valentin & Olmstead, 2004). However, comprehensive studies of floral biology for such plant groups have only been conducted for the *Passiflora* (Kay, 2003), and the tribe Gesnerieae (Martén-Rodríguez *et al.*, 2009). Of these two groups, only the Gesnerieae radiated in the Antilles from a single common ancestor (Smith, 1996; Zimmer *et al.*, 2002; Roalson *et al.*, 2005). Recent field studies have documented both specialized (e.g. hummingbird or bat) and generalized pollination systems (involving hummingbirds, insects and bats), as well as autonomous self-pollination mechanisms in some members of the tribe (Martén-Rodríguez & Fenster, 2008; Martén-Rodríguez *et al.*, 2009). The association of floral phenotypes with particular pollination systems suggests that pollinators have had a significant influence on floral diversification in Gesnerieae. Owing to its insular geographic distribution, monophyly,

great floral diversity, and availability of natural history data, the Gesnerieae makes an exceptional study system to examine trends in floral diversification within a phylogenetic context.

We generated phylogenies using molecular and morphological data and asked the following questions:

- Are pollination systems evolutionarily labile in Gesnerieae?
- Is there evidence for pollinator-mediated selection on floral phenotypes (i.e. convergent or parallel evolution of floral characteristics in response to pollination-system transitions)?
- Does generalization in pollination systems evolve from specialization?
- Is the evolution of reproductive assurance mechanisms associated with particular pollination systems?

Based on the findings of ecological studies, we hypothesized that pollinator-mediated selection underlies transitions in floral traits, regardless of the level of specialization.

Materials and Methods

Study system

The tribe Gesnerieae (family: Gesneriaceae) is distributed across the Antillean islands with a center of diversity in the Greater Antilles and three species that occur in northern South America. Four genera are currently included within the tribe: *Gesneria* – 53 species that display great variation in growth form and floral morphology; *Rhytidophyllum* – 19 species of shrubs with subcampanulate or tubular corollas; *Pheidonocarpa* – a monotypic genus of short shrubs with tubular flowers from Cuba and Jamaica; and *Bellonia* – two species in Cuba and Hispaniola; short shrubs with rotate, white flowers. *Bellonia* was originally classified within the tribe Gloxinieae; however, recent molecular studies provide strong support for *Bellonia* as a member of the Gesnerieae (Roalson *et al.*, 2005).

The pollination systems of 20 Gesnerieae species from the Greater Antilles and St Lucia were characterized in earlier studies from 2003 to 2007 for a total of 602 h of pollinator observations (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez *et al.*, 2009). Species for which anecdotal or unpublished pollination biology data were available were also included. The pollination system of each species and source reference are listed in Supporting Information, Table S1. Voucher specimens for each species are listed in Table S2. Floral phenotypes in Gesnerieae were associated with particular pollination systems in a non-phylogenetically corrected assessment of pollination syndromes as follows: tubular, brightly colored, diurnal flowers are hummingbird-pollinated; campanulate, white or green flowers with nocturnal anther dehiscence are pollinated primarily by bats; subcampanulate flowers with nocturnal

anther dehiscence and corolla color variation are visited by various functional groups of pollinators, including bats, moths and hummingbirds (Martén-Rodríguez *et al.*, 2009). Additional phenotypes include white tubular flowers (*Gesneria humilis*, unknown pollination system), and rotate flowers (e.g. *Bellonia*, associated with buzz pollination by large bees). The principal floral phenotypes are shown in Fig. 1.

Breeding system variation in the tribe Gesnerieae includes variation in the timing of anther dehiscence and stigma receptivity, and in the frequency of self-pollination (Martén-Rodríguez & Fenster, 2008, 2010). Only species with tubular flowers exhibit significant amounts of autonomous self-pollination, although there is great variation among species (4–90% fruit set upon hand self-pollination with floral visitors excluded; Martén-Rodríguez & Fenster, 2010). For campanulate, subcampanulate and rotate-flowered species levels of autonomous self-pollination range between 0 and 9%. The autofertility index (AI) proposed by Lloyd (1992), estimated as $AI = \text{autonomous fruit set} / \text{open pollinated fruit set}$, was used to indicate the degree of autonomous self-pollination. The maturation time of the flower's reproductive organs was recorded while conducting pollinator observations to determine the form of dichogamy (protogyny, protandry), or the lack of it (adichogamy).

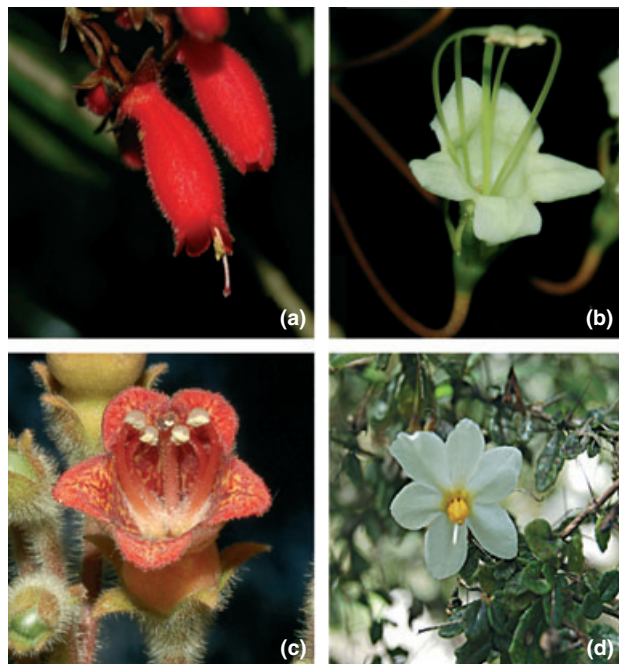


Fig. 1 The four predominant floral phenotypes of Antillean Gesnerieae: (a) *Gesneria decapleura*, tubular red, diurnal flowers – hummingbird-pollinated; (b) *Gesneria pedunculosa*, campanulate nocturnal flowers – bat-pollinated; (c) *Rhytidophyllum auriculatum*, subcampanulate diurnal/nocturnal flowers – pollinated by bats, hummingbirds and moths; (d) *Bellonia aspera* – bee-pollinated.

Taxon sampling

The ingroup included 36 species of the tribe Gesnerieae distributed across all four genera (Table S2). Thirty-two species were collected in the Dominican Republic, Jamaica, Puerto Rico, Cuba, and St Lucia. Tissue samples for *Gesneria rupicola* and *Rhytidophyllum exertum* were obtained from cultivated specimens. Sequences of two *Gesneria* (*G. christii*, *G. humilis*) and three outgroup species (*Gloxinia erinoides*, *Monopyle macrocarpa*, and *Kohleria hirsuta*) were obtained from Genbank. Outgroups were selected based on previous studies that showed strong support for the Gloxinieae as sister tribe to the Gesnerieae (Zimmer *et al.*, 2002; Smith *et al.*, 2004). Although our data set is missing several endemic species from Cuba, it includes good sampling for all other islands, and, more important, it includes a representation of floral variation consistent with the overall proportions of floral phenotypes in the tribe Gesnerieae.

Molecular and morphological data

Leaf tissues were stored in silica gel and DNA was extracted with the Qiagen DNA isolation kit (Qiagen). PCR amplifications and sequencing were performed for nuclear ribosomal ITS and for the nuclear gene *G-CYCLOIDEA* (*GCYC*) for all ingroup species from which tissue samples were available. Methods for DNA extractions, PCR amplifications and sequencing are described in Methods S1.

Morphological characters were scored for all species by examination of herbarium specimens (at US and JBSD), live plants and the literature (Skog, 1976, 1978; Wiehler, 1970, 1983; Kriebel Haehner, 2006; Z Xu & LE Skog, unpublished). A total of 37 morphological characters were scored: 18 characters were associated with vegetative morphology, 16 with inflorescence and flower traits, one with fruit morphology, and one with chromosome number (Tables S3, S4). Chromosome number, three characters of leaf epidermis morphology and one petiole vasculature were scored from the literature (Wiehler, 1970, 1983; Skog, 1976).

Phylogenetic analyses

Maximum-parsimony (MP) analyses for ITS, *GCYC*, morphology and the combined data sets were performed in PAUP 4.0b10 (Swofford, 2002), WinClada (Nixon, 2002), and NONA (Goloboff, 1999). Maximum-likelihood (ML) analyses were performed in GARLI (Zwickl, 2006) using Grid computing (Cummings & Huskamp, 2005) through The Lattice Project (Bazin et al., 2008). A full description of methods used for MP and ML analyses is provided in Methods S1.

Bayesian analyses were conducted for each data set in MrBayes V3.04 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The models of sequence evolution

specified included six substitution rates with base frequencies estimated from the data. First, second and third codon positions were considered separate partitions for the protein coding gene *GCYC*. Site rate variation was modeled using a gamma distribution, and a parameter for the proportion of invariant sites was included. The Markov chain Monte Carlo (MCMC) search was run on four chains for 10 million generations with sampling every 1000 generations. The first 5000 generations were discarded as 'burn-in' after inspection of likelihood plots. For the Bayesian analysis of the total evidence data set, the search used the standard model for morphological data as implemented in MrBayes; nucleotide substitution models and other search terms were as described earlier in this paper.

Character mapping and correlated character evolution

Ancestral character reconstructions were conducted using MP and ML approaches in Mesquite (Maddison & Maddison, 2007), and Bayesian stochastic character mapping (Huelsenbeck *et al.*, 2003) in the software package SIMMAP 1.0 (Bollback, 2006). Optimizations were mostly congruent among different methods, although for some characters there were fewer reversals and multiple transitions along branches never occurred using MP and ML. However, because reversals were overall rare and major results and conclusions were the same under different methods, we only present the Bayesian approach, also used to test for correlated character evolution. Stochastic character mapping analyses were performed using the posterior distribution of 10 000 post-burn-in trees obtained from Bayesian analysis of the total evidence data set. Before running simulations, trees were smoothed using the nonparametric rate smoothing algorithm (NPRS; Sanderson, 1997), as implemented in *TreeEdit* v1.0a10 (Rambaut & Charleston, 2002). Outgroup species were excluded from all character reconstructions. For all simulations we performed 33 realizations per tree, using the default SIMMAP settings as well as two sets of priors selected at random. Selection of priors did not influence the results; thus, for consistency, statistics on character state transitions and correlations are reported for analyses conducted on default priors. For optimization of pollination system, species with no available pollination ecology data were pruned from all trees. The posterior Bayesian expectations for mean and standard deviation of character state transitions are reported for each trait.

The following characters and character states were mapped: pollination system: hummingbird, bat, generalist, bee; corolla shape: tubular, campanulate; subcampanulate, rotate; timing of anther dehiscence and nectar production: diurnal, nocturnal, 'all day'; schedules of anther dehiscence and nectar production were perfectly correlated across all surveyed species, therefore the two traits were jointly optimized: corolla color, white, green, yellow, red, light

yellow with red spots; dichogamy state: adichogamy, protogyny, protandry. Of these characters, corolla shape, color, and dichogamy were included in the matrix used for phylogenetic reconstruction. There has been some contention about the use of phylogenies that are at least partly based on morphological data to reconstruct the evolutionary history of morphological characters (Baker *et al.*, 1998). However, the percentage of characters used in both phylogenetic and ancestral reconstructions was 2% (three out of 154 parsimony informative characters). In additional analyses, the exclusion of these characters did not affect tree topology. Therefore, we used the total evidence dataset, which is the best available phylogenetic hypothesis in terms of resolution and branch support.

Correlated character evolution was evaluated with Bayesian stochastic character mapping in SIMMAP 1.0 (Bollback, 2006). This approach estimates associations among states of discrete characters over a sample of trees (e.g. posterior distribution from Bayesian phylogenetic inference), thus taking phylogenetic uncertainty into account. Character histories are sampled across trees according to their posterior probabilities (Huelsenbeck *et al.*, 2003). The probability of any given character state is proportional to the time the character was in that particular state over the phylogeny. Expected character associations are calculated by multiplying the frequencies of individual states for each combination of two states (Huelsenbeck *et al.*, 2003; Bollback, 2006). Thus, even when evolutionary transitions are rare, if character states tend to co-occur in phylogenies, a signal of association may be detected. The program reports significant results at the 0.05 *P*-level; however, each correlation analysis involves multiple simultaneous comparisons. To correct for multiple comparisons we employed the sequential Bonferroni adjustment. We tested for the association between pollinators (character 1) and floral characteristics (characters 2–5, 7), and for associations among pairs of floral character states.

Results

Phylogenetic analyses

The trees obtained from analysis of ITS and *GCYC* under different methods (MP, ML and Bayesian) resulted in similar topologies (Figs S1–S4). There was no significant incongruence between the two molecular data sets according to the incongruence length difference (ILD) test ($P = 0.166$). Branch support values were also consistent among methods, although posterior probabilities were generally higher, as has been documented in many other studies (Erixon *et al.*, 2003; Ekenas *et al.*, 2007). The analysis of the combined DNA regions provided a more resolved topology than ITS or *GCYC* alone (Figs S5, S6).

Analysis of the morphological dataset resulted in the nine most parsimonious trees (Fig. S7). The topologies obtained

from analysis of molecular and morphological datasets were similar, but marginally significant incongruence was detected (ILD test, $P = 0.09$). Three points of disagreement involve clades with low branch support ($< 50\%$ bootstrap), and the only point of incongruence supported by high bootstrap values was in the placement of *Bellonia*. Morphological data placed *Bellonia* within the tribe Gloxinieae (outgroup), while molecular data placed it within the tribe Gesnerieae. Thus, for all character mapping simulations, we conducted separate analyses including *Bellonia* as an outgroup, and as an ingroup. The conflict did not affect relationships among other ingroup members (hereafter core Gesnerieae clade), nor the interpretation of character mapping and tests of association. The total evidence phylogeny resulted in a better resolved and supported topology than the separate morphology and molecular phylogenies. Parsimony (Fig. S8) and Bayesian searches of the total evidence data set yielded similar results, with a slightly greater resolution of the Bayesian topology (Fig. 2).

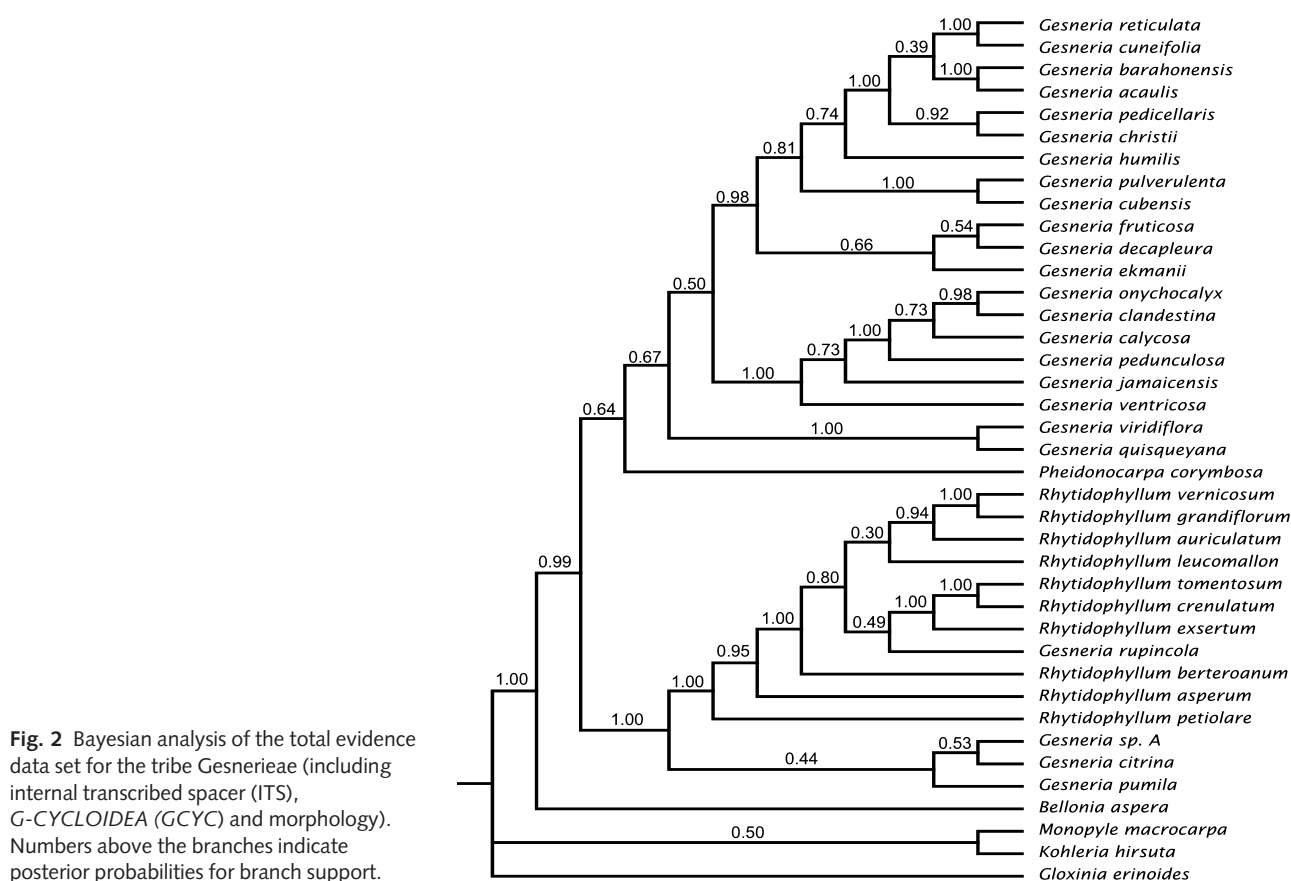
Evolution of floral characters and pollination systems

Our results imply limited lability of pollination-system evolution in the Gesnerieae (Fig. 3). Some simulations resulted in hummingbird pollination and some in bee pollination as the ancestral state for the tribe. However, the basal node for

the core clade of Gesnerieae (excluding *Bellonia*) was reconstructed as having hummingbird pollination (Fig. 3). Posterior expectations (mean (SD)) for pollination-system transitions were: hummingbird to bat (2.6 (0.54)), hummingbird to generalist (1.4 (0.53)), generalist to bat (0.6 (0.50)), generalist to hummingbird (0.7 (0.60)). All other transitions had mean values < 0.5 . These results indicate the most common transitions are leading away from hummingbird pollination.

Analyses suggest tubular corollas are most likely ancestral (Fig. 4). Posterior expectations (mean (SD)) for corolla shape transitions were: tubular to campanulate (2.0 (0.43)), tubular to subcampanulate (4.3 (0.75)), and tubular to rotate (1.0 (0.42)). Reversals to tubular corollas occurred at lower frequencies from campanulate (0.5 (0.65)), and subcampanulate corollas (1.0 (0.95)). Expected means for other transitions were < 0.1 . The evolution of corolla shape was significantly associated with pollination system evolution; shifts to campanulate and subcampanulate flowers generally occur in conjunction with transitions to bat and generalized pollination systems respectively, while transitions to tubular flowers were associated with reversals to hummingbird pollination (Figs 3, 4; Table 1).

Results for timing of anther dehiscence and nectar production indicated that diurnal flowers are most likely ancestral (Fig. 5), with various origins of nocturnal schedules



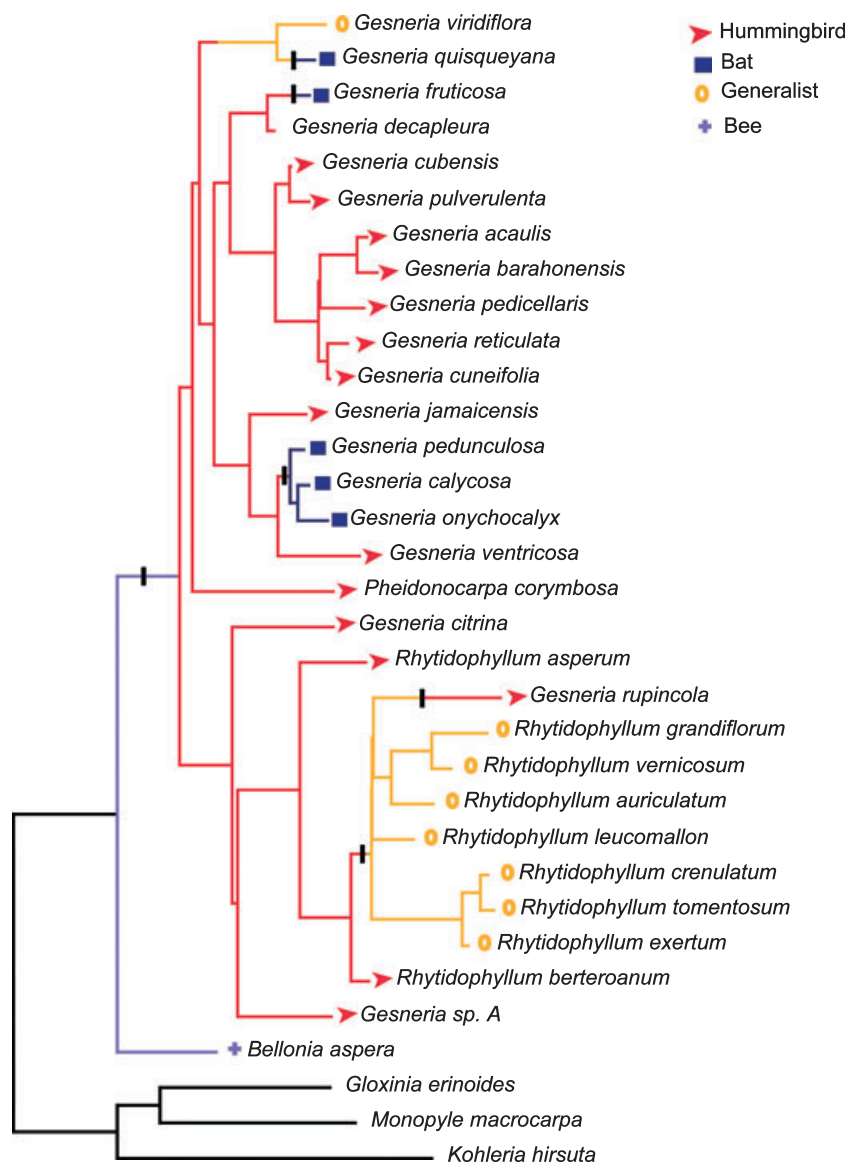


Fig. 3 Pollination system evolution simulated under Bayesian stochastic character mapping. Branch colors and terminal state symbols denote different pollination systems: hummingbird, bat, generalist and bee. Transitions between character states are indicated by changes in color along the branches and black vertical bars.

(4.2 (0.84)), and some reversals (1.74 (1.27)). There was also a transition from nocturnal to 'all day flowers' (1.0 (0.22)) in the lineage of *Rhytidophyllum vernicosum*, a highland species from Hispaniola. The evolution of different floral schedules was significantly associated with changes in pollination system. Specifically, shifts to both bat and generalized pollination were associated with transitions to nocturnal flowers (Figs 3, 5; Table 1).

Analysis of corolla color indicated greater evolutionary lability for this trait (Fig. 6). The most common transitions were from red to: white (4.3 (0.91)), green (1.9 (0.49)), light yellow with red spots (1.1 (0.42)), and pure yellow (0.9 (0.59)). Reversals to red were less frequent and expected in lineages with white (1.21 (0.89)), and light yellow corollas (0.9 (0.32)). Expected means for other transitions were < 0.5. While red and yellow-red flowers were

significantly associated with hummingbird and generalized flowers, respectively; the complete loss of corolla anthocyanines or other pigments responsible for red color was not consistently associated with nocturnal pollination. White or green corollas are characteristic of bat-pollinated species, but these flowers often have dark red markings or trichomes (e.g. *Gesneria fruticosa* and *Rhytidophyllum petiolare*; Figs 3, 6).

Significant statistical associations between pollination system and floral character states were found for all pollination syndrome characters (Table 1). There were also significant correlations among floral character states, such as corolla shape with color and timing of anthesis (Table 2). The associations among characters generally correspond with traits that evolve together under the pollination syndrome concept. For example, tubular flowers are positively

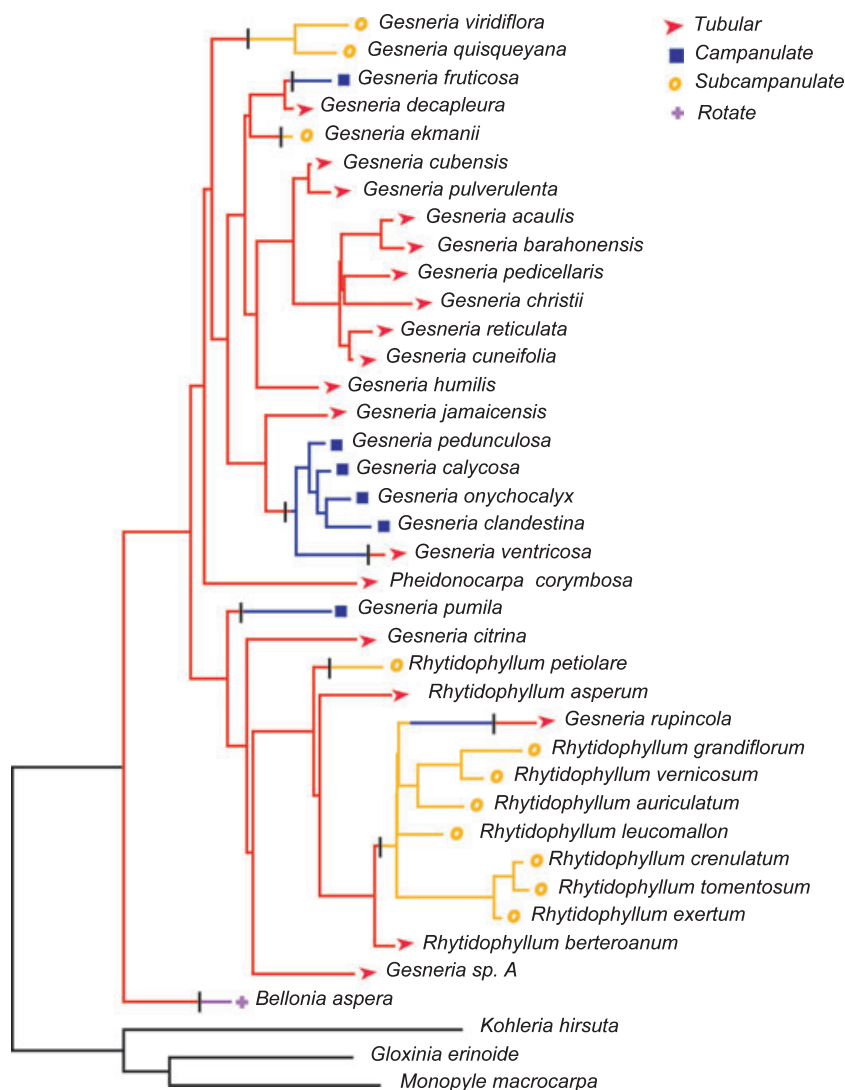


Fig. 4 Evolution of corolla shape simulated under Bayesian stochastic character mapping. Branch colors and terminal state symbols denote different shapes: tubular, campanulate, subcampanulate and rotate. Transitions between character states are indicated by changes in color along the branches and black vertical bars.

associated with red color and diurnal anthesis (i.e. hummingbird pollination syndrome), while subcampanulate corollas were associated with nocturnal anthesis and generalized pollination.

Stochastic mapping of dichogamy indicated limited lability for this trait. Posterior expectations for the number of transitions (mean (SD)) were as follows: protogyny (i.e. where female organs develop first) to protandry (i.e. where male organs develop first) = (2.3 (0.79)), and protandry to protogyny = (0.8 (0.83)). Expected means for transitions to and from adichogamy were low (mean < 0.5). Dichogamy shifts were not associated with pollination system (Figs 3, 7; Table 1).

Owing to the large proportion of species with missing data for autonomous selfing and pollen limitation, character histories were not reconstructed for these traits. However, based on the available data, the ability to set seed autonomously occurs in three separate hummingbird-pollinated lineages (Fig. 8). Likewise, pollen limitation occurs in three

separate lineages that include hummingbird- and bat-pollinated species. By contrast, species with generalized pollination or autonomous breeding systems do not exhibit pollen limitation (Fig. 8).

The geographic distribution of species in the phylogeny suggests dispersal between islands has been common (Fig. 8). Every well supported clade in the phylogeny has species from more than one island and some species have populations across multiple islands. Further biogeographical analyses were not attempted here since better sampling of species from Cuba would be necessary for that purpose.

Discussion

Organisms on islands experience unique selective regimes that may lead to unconventional changes in ecologically important traits. For example, the majority of flowers in oceanic islands have unspecialized floral phenotypes (Carlquist, 1974; Webb & Kelly, 1993), suggesting that

Table 1 Correlation statistic *D* and *P*-values for tests of association between pollination systems and floral traits evaluated using SIMMAP 1.0 (Bollback, 2006)

	<i>D</i> -value (<i>P</i> -value)		
	Hummingbird	Bat	Generalist
Flower shape			
Tubular	0.176 (< 0.001)	−0.07 (0.003)	−0.116 (0.005)
Campanulate	−0.059 (0.009)	0.082 (0.005)	ns
Subcampanulate	−0.115 (0.001)	ns	0.142 (0.004)
Rotate	ns	ns	ns
Color			
White/green	−0.09 (0.006)	0.08 (0.005)	−0.04 (0.02)
Yellow/red	−0.09 (0.002)	ns	0.13 (0.003)
Red	0.179 (0.001)	−0.06 (0.01)	−0.09 (0.006)
Yellow	ns	ns	ns
Timing			
Diurnal	0.17 (0.001)	−0.07 (0.006)	−0.12 (0.004)
Nocturnal	−0.16 (0.002)	0.08 (0.008)	0.11 (0.006)
Both	ns	ns	ns

Associations including bee and small insect pollination were nonsignificant and thus *D*-values are not shown below. Negative associations are indicated by a minus sign. *P*-values are reported for associations that were found significant by the program, with bold values representing significant associations after sequential Bonferroni adjustment for multiple comparisons. ns, not significant.

shifts to generalization have been common in the evolutionary history of island plants. By contrast, transitions from generalized to specialized or between specialized pollination systems are the most frequently reported patterns in studies of floral evolution conducted primarily on mainland plant taxonomic groups (reviewed in Weller & Sakai, 1999; Fenster *et al.*, 2004; Tripp & Manos, 2008). This study provides the first evidence supported by pollination ecology data, for evolutionary transitions towards generalized pollination in a plant group from the Caribbean islands. At the same time, there is evidence for diversification of specialized hummingbird and bat-pollinated lineages. Below we examine patterns of pollination and breeding system evolution for the tribe Gesnerieae, and discuss how simultaneous study of both aspects of plant reproduction within the context of phylogeny can provide important insights for floral evolution.

Floral evolution

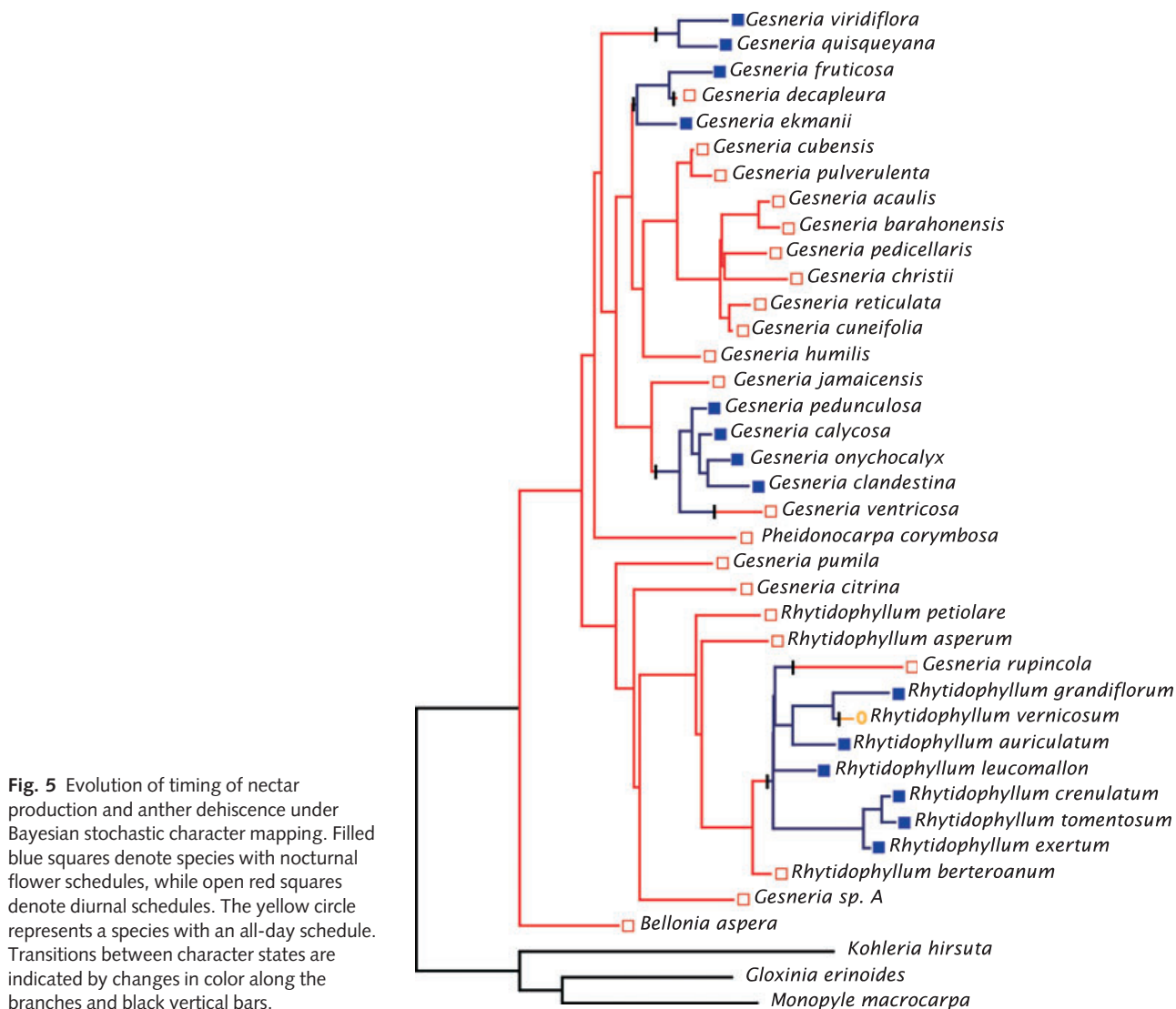
Suites of correlated floral traits or 'pollination syndromes', are generally believed to reflect convergent selection pressures exerted by one functional group of pollinators (Faegri & van der Pijl, 1978). The results of this study support our previous findings in support of classic bat and hummingbird pollination syndromes in a nonphylogenetically corrected study of the Gesnerieae (Martén-Rodríguez *et al.*, 2009). Additionally this study revealed that in Antillean Gesnerieae,

pollination system transitions occur either by switching pollinator functional groups (for example, hummingbird to bat), or by adding different functional groups (e.g. bats and insects).

The evolution of bat pollination in Gesnerieae involves changes in flower color, timing of anther dehiscence (Fig. 4), and timing and quantity of nectar production. The latter trait was not mapped onto the phylogeny because of the small number of species for which daily nectar production was quantified. However, where these estimates are available, nectar volume averages 12.5 µl (± 3.99, *n* = 3) for hummingbird-pollinated species, 75.2 µl (± 14.85, *n* = 2) for bat-pollinated species, and 67.1 µl (± 7.55, *n* = 3) for generalists (Martén-Rodríguez & Fenster, 2008; Almarales-Castro & S Martén-Rodríguez, unpublished). High nectar production and floral scent are considered important attractants in bat-pollinated flowers, including members of the Gesneriaceae family (Sazima *et al.*, 1999; Tschpka & Dressler, 2002); however, tribe Gesnerieae species have no distinguishable floral scent. Lack of scent in bat-pollinated Gesnerieae may be indicative of recent origins of chiropterophilous flowers from odorless hummingbird-pollinated ancestors and illustrates how fit to classic pollination syndromes is often incomplete due to genetic or historical constraints.

The evolution of generalized pollination by bats, moths and hummingbirds in Gesnerieae was associated with the evolution of subcampanulate corollas. Other floral traits in these species probably reflect adaptation to both hummingbirds and nocturnal pollinators. For instance, broad corolla openings, schedules of anther dehiscence and nectar production reflect selection by nocturnal pollinators. However, the constriction above the nectar chamber and red markings on yellow corollas appear to reflect selection by hummingbirds. In particular, the corolla constriction may promote contact of the hummingbird's bill with the reproductive organs of the flower (Martén-Rodríguez *et al.*, 2009), an idea that needs to be empirically tested. In generalized Gesnerieae, hummingbird visits occur both in late afternoon, when nectar production starts, and during early morning hours (Martén-Rodríguez *et al.*, 2009). However, since pollen is mostly unavailable until c. 18:00 h, hummingbirds should be most effective at dawn, particularly when nocturnal pollination has failed. Under this scenario, generalization may provide a mechanism for reproductive assurance where pollinator service by bats is low.

Studies of other plant species that share bat and hummingbird pollination are inconclusive as to whether intermediate traits represent transitional phenotypes, or phenotypes adapted to both functional pollinator groups (e.g. *Abutilon*, Buzato *et al.*, 1994; *Syphocampylus sulfureus*, Sazima *et al.*, 1994). It has been suggested that a stage of greater generalization is likely to occur between transitions among specialized pollination systems (Baker, 1963; Wilson



et al., 2006). The fact that even specialized flowers are often visited by a number of the potential pollinators present in a community would allow selection by different floral visitors to operate at a given time and space (Baker, 1963). During a transitional stage, floral phenotypes would reflect these different selective pressures, as suggested for some species of *Penstemon* (bird- and bee-pollinated, Wilson *et al.*, 2006). However, under certain ecological conditions a particular pollinator group may become more important and drive floral specialization in an alternate direction (Baker, 1963).

In Gesneriaceae, floral change driven by the pollinator environment is exemplified in the lineage of generalists, including *Rhytidophyllum grandiflorum* and *R. vernicosum*. Both species occur at high elevations (> 1500 m) in the Dominican Republic, where nectar-feeding bats are absent or rare. While *R. grandiflorum* maintains nocturnal schedules of anther dehiscence and nectar production, *R. vernicosum* shows a mixed phenotype with diurnal and

nocturnal schedules (i.e. plants and flowers within plants vary in the amount of red pigmentation in corollas, and in the schedules of nectar production and anther dehiscence). *R. vernicosum* has a higher frequency of hummingbird visitation than other generalists, and successful pollen deposition is facilitated by strong corolla curvature. Moths also contribute to pollination in this species (Martén-Rodríguez *et al.*, 2009; S Martén-Rodríguez, unpublished). Overall, these findings suggest that *R. vernicosum* may represent a transitional generalized stage reverting to hummingbird pollination.

Based on the current phylogenetic hypothesis and stochastic mapping of characters, reversals to hummingbird pollination are rare in Gesneriaceae. This result parallels findings of other studies where pollinator transitions are labile only in certain directions, for example, bee to hummingbird in *Costus* (Kay *et al.*, 2005) and *Penstemon* (Wilson *et al.*, 2007), ornithophilous to chiropterophilous flowers in

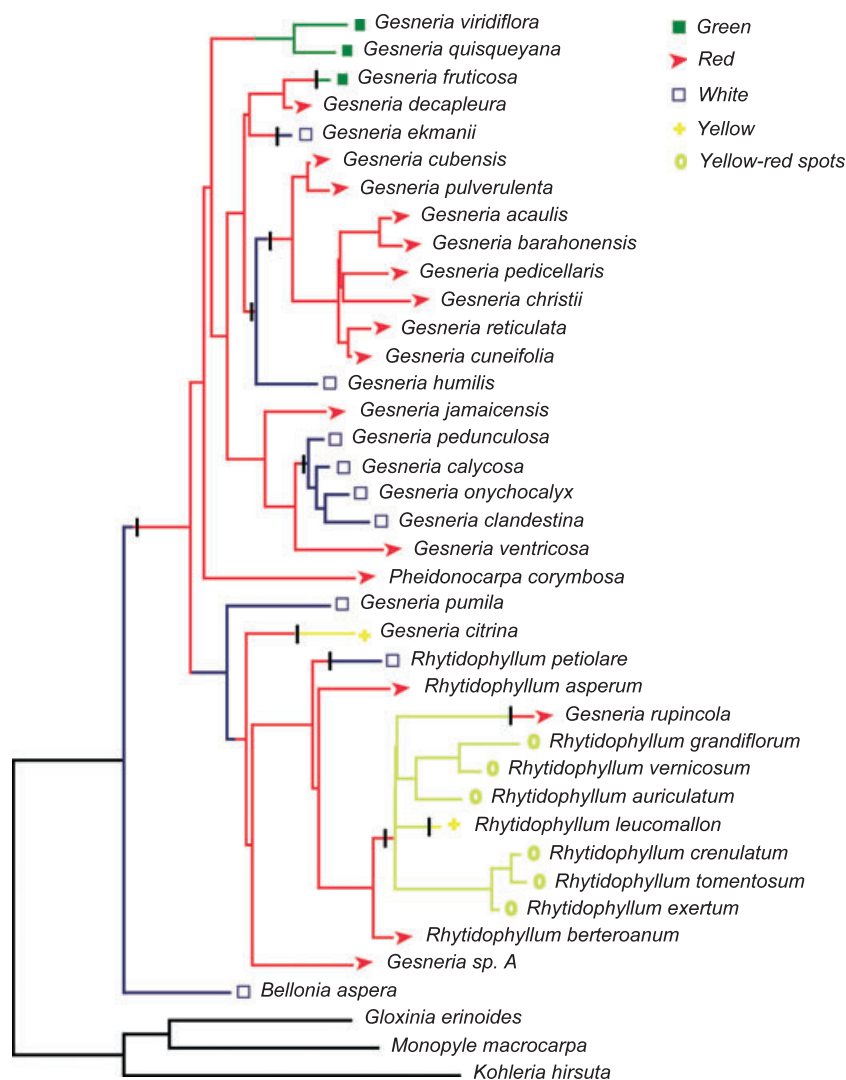


Fig. 6 Evolution of timing of corolla color under Bayesian stochastic character mapping. Terminal color states are represented by different symbols. Transitions between character states are indicated by changes in color along the branches and black vertical bars.

Sinningieae (Perret *et al.*, 2003), and diurnal to nocturnal pollination in *Ruellia* (Tripp & Manos, 2008). Possible causes for these unidirectional trends are: environmental constraints (e.g. the effectiveness of available pollinators; Wilson *et al.*, 2007), or internal constraints (e.g. physiological limitations; Rausher, 2008). For example, the evolution of nocturnal pollination frequently involves loss of red pigmentation. This floral transition has been associated with 'loss of function' mutations in the pathway of anthocyanin production (Mol *et al.*, 1998), which makes loss of red color difficult to regain (Whittall *et al.*, 2006; Rausher, 2008). However, physiological constraints do not satisfactorily explain the case of Gesnerieae, since the ability to produce red floral pigments is not lost in all lineages of generalist and bat-pollinated species (Figs 1, 6). The directionality of transitions in Gesnerieae is likely determined by a combination of historical factors (i.e. hummingbird-pollinated flowers are ancestral), environmental constraints (e.g. low hummingbird visitation), and internal constraints (e.g.

the ability to evolve self-pollination mechanisms for reproductive assurance and inbreeding history).

Breeding system evolution

Autonomous self-pollination is thought to provide reproductive assurance in many angiosperm species across a wide range of floral morphologies and pollination systems (Lloyd, 1992; Fenster & Martén-Rodríguez, 2007). In Gesnerieae, autonomous self-pollination occurs in three independent lineages, all of which are hummingbird-pollinated (Fig. 7). Ecological studies suggest that this association was promoted by the low and unpredictable pollinator service by hummingbirds in the Caribbean islands. Three findings support this assertion: first, hummingbird-pollinated species have the lowest frequencies of pollinator visitation (mean number of visits per flower $d^{-1} = 1 \pm 1.5$ SE, $n = 9$) when compared with bat-pollinated (2 ± 1.8 , $n = 5$) and generalist species (13 ± 1.8 , $n = 5$), where n = number of observed

Table 2 Correlation statistic D and *P*-values for tests of association among floral traits evaluated using SIMMAP 1.0 (Bollback, 2006)

	Color			Anthesis/Nectar production	
	White/green	Yellow-red	Red	Diurnal	Nocturnal
Flower shape					
Tubular	−0.06 (0.007)	−0.01 (0.01)	0.165 (0.001)	0.19 (0.001)	−0.18 (0.001)
Campanulate	0.07 (0.008)	ns	−0.05 (0.01)	−0.06 (0.01)	0.06 (0.01)
Subcampanulate	ns	0.123 (0.005)	−0.106 (0.006)	−0.13 (0.003)	0.13 (0.003)
Color					
White/green				−0.05 (0.02)	0.06 (0.02)
Yellow-red				−0.1 (0.005)	0.1 (0.006)
Red				0.16 (0.001)	−0.15 (0.002)
Yellow				ns	ns

Negative associations are indicated by a minus sign. *P*-values are reported for associations that were found significant by the program, with bold values representing significant associations after sequential Bonferroni adjustment for multiple comparisons. ns, not significant.

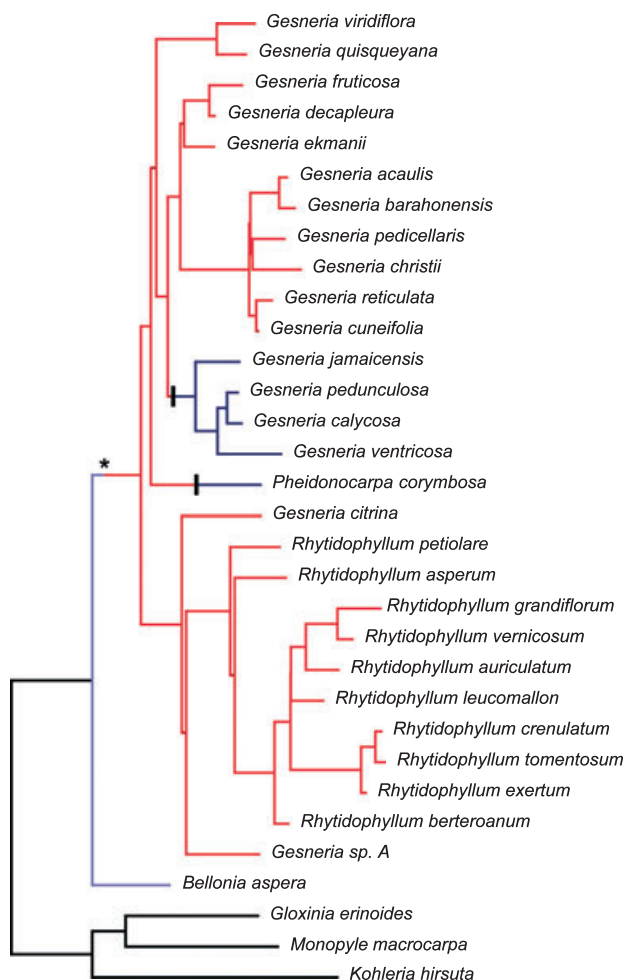


Fig. 7 Evolution of dichogamy under Bayesian stochastic character mapping. A hypothesized origin of protogyny (red) is indicated by an asterisk above the branch; two hypothesized origins of protandry (blue) are indicated by vertical bars.

species (Martén-Rodríguez *et al.*, 2009); second, significant pollen limitation (higher fruit set of hand-pollinated vs open-pollinated plants) was detected only in specialized

species (Martén-Rodríguez & Fenster, 2010); third, autonomous self-pollination provides reproductive assurance in three out of four studied hummingbird-pollinated Gesnerieae (Martén-Rodríguez & Fenster, 2010). Overall, these findings suggest that inadequate pollinator service underlies the evolution of autonomous self-pollination in ornithophilous Gesnerieae as a strategy to mitigate pollen limitation and ensure seed production when vector-mediated pollination fails. This rationale, however, does not explain why bat-pollinated Gesnerieae, which are also pollen-limited, have not evolved reproductive assurance mechanisms.

Could the observed pattern of breeding system evolution be the result of differential expression of dichogamy among hummingbird- and bat-pollinated species? Protogyny provides a more intuitive mechanism for reproductive assurance because self-pollination can occur at the end of the receptivity period (Bertin & Newman, 1993; Mallick, 2001). However, although protogyny tends to be associated with self-compatibility (Routley *et al.*, 2004), a general association of protogyny with autonomous selfing mechanisms has not been demonstrated (Fenster & Martén-Rodríguez, 2007). In Gesnerieae, autonomous selfing has evolved only in protogynous lineages, but the evolution of animal pollination systems is not associated with shifts in dichogamy (e.g. origins of protandry occur both in hummingbird- and bat-pollinated lineages, Fig. 7). Thus, the results indicate that expression of dichogamy is not responsible for variation in reproductive assurance mechanisms among Gesnerieae species.

An alternative explanation is that autonomous pollen transfer is related to flower shape. In tubular corollas the reproductive organs are in close proximity, making autonomous deposition of self-pollen on stigmas more likely. This idea is supported by a study of South American *Schizanthus* (Solanaceae), where autonomous self-pollination has evolved only in tubular-flowered species pollinated by hummingbirds or moths (Perez *et al.*, 2006). Thus, the positioning of the reproductive organs in narrow corollas may constitute a

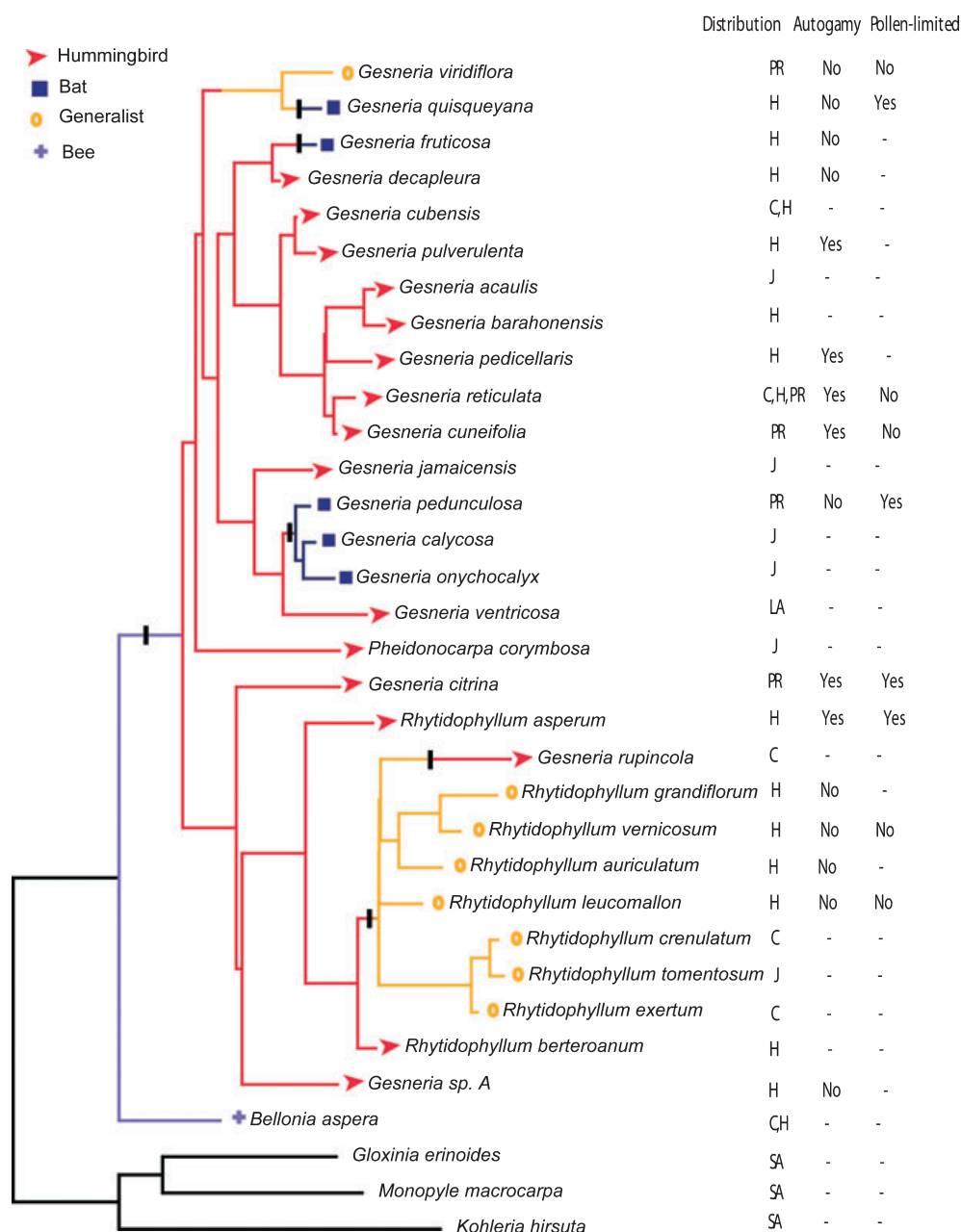


Fig. 8 Geographic distribution, autonomous self-pollination and presence or absence of pollen limitation in Gesneriaceae. Distribution codes: C, Cuba; J, Jamaica; H, Hispaniola; PR, Puerto Rico; LA, Lesser Antilles; SA, South America. Autonomous selfing index was calculated as $AI = 1 - (\text{fruit set bagged} / \text{fruit set outcrossed flowers})$; yes, $AI > 0.17$; no, $AI < 0.9$. Significant pollen limitation was determined in field experiments comparing pollen-supplemented and open-pollinated plants.

preadaptation to the evolution of the reproductive assurance mechanisms by increasing the precision of self-pollen deposition (Mazer & DeLesalle, 1998).

A unified view of pollination and breeding system evolution: what is special about islands?

Pollination systems in islands show a great proportion of generalized interactions (Olesen *et al.*, 2002), and high

frequency of wind pollination (Bernardello *et al.*, 2001). Pollinator-depauperate faunas on islands are thought to be responsible for these trends. First, reduced interspecific competition may cause island species to have broader feeding niches than their mainland relatives (Olesen *et al.*, 2002). For example, in the Dominican Republic, hummingbirds are represented by only three species, one of which is so small that it cannot access nectar from typical ornithophilous flowers. Thus, a single hummingbird species

may be responsible for the pollination of all ornithophilous plant species in certain communities. Additionally, and perhaps related to low species diversity, visitation frequencies on islands are often lower than in mainland regions (Linhart & Feinsinger, 1980; Martén-Rodríguez *et al.*, 2009).

Under conditions of low pollinator service, natural selection should favor reproductive strategies that reduce the risk of pollination failure on islands. For instance, the ability to self may partly explain the maintenance and diversification of hummingbird lineages in the Antilles. The results of this study support the idea that low diversity of pollinator species on islands select for generalization and autogamy as reproductive assurance mechanisms. Our findings highlight the importance of simultaneously studying pollination and breeding system evolution to achieve a comprehensive understanding of the processes underlying floral diversification.

Acknowledgements

We are grateful to Abel Almarales, Tirzah Breslar, Teodoro Clase, Julie Cridland, Xin-Sheng Chen, Michel Faife, Bryan Gough, Daniel Growald, Joe Hereford, Carlo Moreno, Brígido Peguero, and Daniel Stanton for help conducting field work. We thank Stacy DeWitt Smith and Paul Wilson for insightful revisions on earlier versions of this manuscript. We thank the Institute of Jamaica, and Jardín Botánico de Santo Domingo for logistical support and John L. Clark for contributing tissue samples of *Rhytidophyllum crenulatum*. We thank Pedro Acevedo, David Erickson, Chris Huddleston, Akito Kawahara, and Ken Wurdack for support and advice provided during various stages of the project. Funding was provided by The Gesneriad Society, The Explorers Club-Washington Group, Graduate Women in Science, Sigma Xi, Bamford Fund and BEES Program (University of Maryland), Smithsonian Institution, and NSF DDIG 0710196 to S.M.R. and C.B.F.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Strict consensus of eight most parsimonious trees (length = 453; CI = 78; RI = 80) from analysis of the ITS dataset.

Fig. S2 Bayesian analysis of the ITS dataset for the tribe Gesnerieae.

Fig. S3 Strict consensus of six most parsimonious trees (length = 114; CI = 94; RI = 95) from analysis of the *G-CYC* dataset.

Fig. S4 Bayesian analysis of the *G-CYC* dataset for the tribe Gesnerieae.

Fig. S5 Strict consensus of 1200 most parsimonious trees (length = 315; CI = 78; RI = 81) from analysis of the combined ITS + *G-CYC* dataset.

Fig. S6 Bayesian analysis of the combined ITS + *G-CYC* dataset for the tribe Gesnerieae.

Fig. S7 Strict MP consensus of nine most parsimonious trees (length = 169; CI = 33; RI = 71) from analysis of Gesnerieae morphology dataset.

Fig. S8 Strict consensus of 27 most parsimonious trees (length = 646) from analysis of the total evidence dataset (ITS, *G-CYC* and morphology) dataset for the tribe Gesnerieae.

Methods S1 Extended methods for DNA sequencing and phylogenetic analyses.

Table S1 Pollination systems of 39 Gesneriaceae species from the Caribbean islands and Costa Rica.

Table S2 List of specimens sampled for molecular phylogeny.

Table S3 Morphological characters and character states used in phylogenetic analyses of the tribe Gesnerieae.

Table S4 Morphological data matrix of 36 Gesneriaceae species and three Gloxinieae outgroups.

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