ABSTRACT

Title of Dissertation: EVOLUTION OF POLLINATION AND BREEDING SYSTEMS OF ANTILLEAN GESNERIACEAE.

Silvana Martén Rodríguez, Doctor of Philosophy, 2008

Directed By: Dr. Charles B. Fenster, Biology Department

Islands serve as one of our most important model systems for understanding the role of the environment on character evolution and diversification. This study examined the role of pollinators on the evolution of floral traits and breeding system on the tribe Gesnerieae (Gesneriaceae), a monophyletic radiation from the Antilles that encompasses great floral diversity. Pollinator observations were conducted over 5 years (2003-2007) for 19 species of Gesnerieae across multiple islands. Pollination systems include specialized systems (i.e. one functional group of floral visitors, e.g. hummingbird or bats) and generalized systems (e.g. various functional groups, e.g., bats, birds and insects). Hummingbird-pollinated species have the lowest frequencies of pollinator visitation (mean number of visits per flower/ per day =1 ± 1.5 SE) compared to bat-pollinated (mean= 2 ± 1.8) and generalist species (mean=13 ± 1.8). A multivariate analysis of floral traits provides strong support for correlated sets of floral traits associated with bat and hummingbird pollination supporting the pollination syndrome concept (the notion that flowers evolve integrated phenotypes in response to selection by the most important pollinators). A two-year pollen limitation study of nine species was conducted to evaluate how differences in visitation among different pollination system influenced plant female reproductive success; this study resulted in significant pollen limitation for specialized species with low visitation only (bat and hummingbird-pollinated). Furthermore, emasculation experiments demonstrated that ornithophilous species use autonomous self-pollination as a reproductive assurance mechanism. A survey of potential autonomous self-pollination for 15 Gesnerieae representative of all floral phenotypes supported an association between this breeding system hummingbird-pollinated tubular-flowered species. Last, phylogenetic analyses of two nuclear DNA regions (ITS and *GCYC*) and a morphological data set revealed that bat and generalized pollination evolved from hummingbird pollination. Furthermore, autonomous self-pollination originates only in ornithophilous lineages of Gesnerieae. Overall these results provide evidence for the evolution of generalization and autonomous breeding systems, as two alternative reproductive strategies in response to inadequate hummingbird pollination service in insular ecosystems. This study underscores the utility integrating the study of pollination and breeding systems within a phylogenetic context to provide further insights into the ecological conditions that can influence floral evolution.

EVOLUTION OF POLLINATION AND BREEDING SYSTEMS OF ANTILLEAN GESNERIACEAE.

By

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CHAPTER 1

Pollination ecology and breeding systems of five *Gesneria* species from Puerto Rico.

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Abstract

The genus *Gesneria* diversified in the Greater Antilles giving rise to various floral designs corresponding to different pollination syndromes. The goal of this study was to The study was conducted in Arecibo and El Yunke National Forest, Puerto Rico, between 2003 and 2007. Floral visitors were documented by human observers and video cameras. Floral longevity and nectar production were recorded for the five study species. Tests for self-compatibility and autonomous selfing were conducted through hand-pollination and bagging experiments. Floral phenology and nectar production schedules agree with nocturnal (in bell-shaped flowered *G. pedunculosa* and *G. viridiflora* subsp. *sintenisii*) or diurnal pollination syndromes (in tubular-flowered *G. citrina*, *G. cuneifolia* and *G. reticulata*). Nectar concentration is consistently low (eight to thirteen percent) across species. *Gesneria citrina* and *G. cuneifolia* are exclusively pollinated by hummingbirds, while *Gesneria reticulata* relies mostly on autonomous self-pollination, despite having classic ornithophilous flowers. A variety of floral visitors were recorded for the two bell-shaped flowered species; however, not all visitors have the ability to transfer pollen. Bats are the primary pollinators of *G. pedunculosa,* with bananaquits probably acting as secondary pollinators. For *G. viridiflora* subsp. *sintenisii,* both bats and hummingbirds contact the flower's reproductive organs, thus, we consider this species a generalist despite its nocturnal floral syndrome. All species are self-compatible but only tubular-flowered *Gesneria* are capable of autonomous self-pollination.

The visitation patterns described in this study fit the predicted hummingbird and bat pollination syndromes and support both specialization and generalization of pollination systems in Puerto Rican *Gesneria*. Specialization is associated with low pollinator visitation, particularly by hummingbirds, which may explain the occurrence of autonomous selfing mechanisms in tubular-flowered species.

Key words: Autonomous selfing, bat pollination, breeding systems, *Gesneria*, hummingbird pollination, Puerto Rico.

Introduction

Pollination specialization has long been considered an important process underlying the evolution of floral diversity. Consequently floral traits have been viewed as adaptations to attract specific pollinators and to enhance efficiency of pollen transfer and outcrossing (Darwin, 1862; Stebbins 1970). In recent years, this classic view of specialization has been a subject of contention (Waser et al., 1996; Johnson and Steiner, 2000; Fenster et al., 2004). Waser and colleagues (1996) argue that most plant species have generalized visitation patterns and that floral visitors rarely specialize on particular food sources. While it is true that generalization at the community level is common in temperate regions (e.g. Herrera, 1998; Gomez, 2002), specialization in functional groups of pollinators is also widespread (Armbruster et al., 2000; Fenster et al., 2004). More knowledge of the floral biology of plants in tropical regions is necessary to achieve a better understanding of the overall ecological and evolutionary patterns of specialization (Johnson and Steiner, 2000).

Island plants may provide further insights into the evolution of specialization and generalization of pollination systems. Islands tend to have lower abundance and diversity of many important pollinator groups (Carlquist 1974, Barrett 1996), thus, the particular patterns of abundance, composition and behaviour of floral visitors on islands may create selective environments different from those that flowers are exposed to in mainland regions (Barrett, 1996; Armbruster and Baldwin, 1998). Two literature reviews suggest that islands generally have a greater representation of species pollinated by generalist insects compared to mainland regions (Carlquist, 1974; Barrett, 1996). However, the floral diversity of plant radiations from some tropical islands suggests that pollinator specificity may be a common feature of some insular plant taxa (e.g. Hawaiian mints, Lindqvist and Albert, 2002; Hawaiian Lobeliads, Lammers and Freeman, 1986). Here we document the pollination and breeding systems of five *Gesneria* species belonging to one such plant group, the tribe Gesnerieae from the Antillean islands (Skog, 1976).

Given that floral structures have functional significance for both pollination and breeding systems, simultaneous study of both will lead to a better understanding of the ecological and evolutionary processes that drive floral trait diversification (Holsinger, 1996; Barrett et al., 1996; Barrett, 2003). Plants on islands may benefit from having selfing breeding systems, particularly during colonization and establishment or when pollinator abundances are low (Baker, 1955; Gonzales-Diaz and Ackerman, 1988). However, reduced genetic diversity and higher levels of inbreeding depression have been found associated with selfing in some insular plant species (e.g., Naito et al., 2005). Consequently, traits that promote outcrossing may be selected for after island colonization (Carlquist, 1974; Barrett, 1996).

This study provides the first step in the characterization of pollination and breeding systems of the tribe Gesnerieae (Family Gesneriaceae). The tribe is a monophyletic clade of the family Gesneriaceae distributed primarily across the Antilles (Zimmer *et al.,*2002). We conducted field studies on five *Gesneria* species from the island of Puerto Rico in order to: (1) characterize their floral biology, including morphology, floral phenology and nectar production, (2) document floral visitor assemblages and behaviour of pollinating and non-pollinating flower visitors; and (3) characterize breeding systems (dichogamy, self-compatibility, autonomous selfing) and assess them in the context of the pollination system.

Materials and Methods

Study sites and species: According to Zimmer *et al.* (2002) and our preliminary phylogenies, the genus *Gesneria* is paraphyletic and our study species belong in at least two different clades. *Gesneria reticulata* and *G. cuneifolia* are sister species within a clade that comprises most species in the genus; *Gesneria citrina* groups within the genus *Rhytidophyllum*, while *G. pedunculosa* and *G. viridiflora* belong in two clades that have ambiguous placement in our preliminary phylogeny. The pollination biology of *Gesneria pedunculosa, G. citrina* and *G. cuneifolia* was studied in the forests surrounding the Arecibo Observatory (18°20′36.6″N, 66°45′11.1″W ca. 300 m.s.l.) in Puerto Rico. These three species are endemic to the island. The Arecibo Observatory is located in the northern karst region of the island, and is characterized by a topography of limestone hills and valleys with an underground drainage structure that includes extensive cave systems. Some of the caves in the region are known to host large bat populations, including the nectarivorous *Monophyllus redmanii*.

Gesneria viridiflora subsp. *sintenisii* and *G. reticulata* were studied in El Yunque mountain, which rests within the boundaries of the Caribbean National Forest (18°19′ N, 65°47′ W). Plants of *G. viridiflora* subsp. *sintenisii* occur along rainforest streams, between 700-1000 m. This species is also a Puerto Rican endemic. *Gesneria reticulata* has a narrow distribution in Puerto Rico, where it has only been collected along route 191, between kilometres 9 and 11; however this species occurs in Cuba and Hispaniola.

Pollinator observations: Characterization of the pollinator fauna of five Gesnerieae species was accomplished in January and June of 2003 and December through March of 2005 - 2007. Floral visitors were recorded from dawn to dusk for 0.5 hour periods by human observers and 1.5 hour periods by video cameras. Observations were performed on 22 - 40 individuals per species, at different times of the day and on scattered days throughout the flowering period; total observation time per species is listed in Table 3. Nocturnal observations were conducted regardless of the floral syndrome to avoid biasing the sampling towards the expected pollinators; however, these observations were limited to eight hours in species with diurnal nectar production. For all nocturnal observations video cameras with infrared night vision were used (SONY Handycam DCR-HC42 and DCR-TRV350). The identity of visitors was recorded to the lowest possible taxonomic level as determined by examination of the video recordings. When possible, insect visitors were also collected, however, collections were not made during observation time to prevent altering natural visitation rates. Time and duration of the visit, number of flowers, type of reward (nectar or pollen), and contact with anthers or stigmas were recorded. Visitation rates per flower per hour were calculated for each species as the total number of visits divided by the observation time, divided by the number of observed flowers. We multiplied the estimate by 12 to obtain diurnal or nocturnal pollination rates per 12 hour day or night, according to the visitation schedule of the pollinator.

Nectar production and floral phenology: To determine the schedule of nectar production one to three flower buds from 12 - 32 plants were bagged and nectar was measured every six hours for two days. Nectar amounts were measured using a 50 µL syringe (Hamilton, NV, USA), and 5 µL capillary tubes for species with low production. Nectar concentration was measured once or twice per flower using a hand-held refractometer (Sugar/Brix Refractometer, 0–32% w/ATC, Sper Scientific, Scottsdale, Arizona, USA). All nectar data were collected in December 2006 and January 2007. Differences in nectar volumes among time periods and sugar concentration among four species were tested with ANOVA using the GLIMMIX procedure in SAS version 9.1.3 (SAS Institute, 2004). A repeated measures model was specified for the analysis of time periods because the same flowers were measured at different times. *Gesneria reticulata* was excluded from the analysis because only three out of 32 flowers we tested produced any measurable amount of nectar.

We studied the floral phenology of all species by following 15- 20 flowers from bud until senescence in January 2005. We checked flowers every three hours to determine the timing of anther dehiscence. For the two species with flowers, once a range of times was established, we followed flowers every hour to document the specific time of pollen release. This was not done for tubular-flowered species because anthers are often positioned within the floral tube when they dehisce and it was difficult to assess this trait without damaging the flower. Stigma receptivity was determined four times a day (0600 h, 1200 h, 1800 h and 0000 h) by adding a drop of hydrogen peroxide and checking for bubble formation (Kearns and Inouye, 1993) on different flowers of known age.

Breeding systems: Hand-pollination experiments were used to test for self-incompatibility systems. Twelve plants per species were used for each of two treatments: hand self-pollination, and hand outcross-pollination. Flower buds were covered with wedding veil bags and stigmas were pollinated by rubbing anthers of donor flowers onto the stigmas of recipient flowers. Outcross pollen was collected from two to three donors growing at least seven metres away to reduce the chances of crossing with pollen from genetically related individuals. Self-pollinations were performed using pollen donor flowers from the same plant.

To evaluate potential for autonomous selfing, the ability to set seed in the absence of pollinators, all flower buds developing for a period of one to two weeks in 15 - 28 plants were bagged. Fruit set was determined two months later and seed mass was estimated in the lab. An autofertility index was calculated by dividing the fruit set of bagged flowers by the fruit set of hand-outcrossed flowers (Lloyd and Schoen 1992). Tests for apomixis that occurs when no pollen deposited on stigmas were conducted by emasculating and bagging two to four flower buds from seven to ten individuals per species.

We tested for differences in fruit set (fruits/flowers) and seed mass among hand-self, hand outcross and bagging with ANOVA using the GLIMMIX procedure in SAS (SAS Institute, 2004). Pairwise differences were tested specifying the Tukey adjustment for multiple comparisons. Seed mass of bagged flowers was not compared to hand-pollination treatments in *G. pedunculosa* and *G. viridiflora*, because most plants did not set fruit. Gesnerieae fruit capsules contain many small seeds (300 - > 3,000), making counting of all seeds impractical. However, significant correlations between seed number and seed mass (n=30 capsules for each of five species, r =0.87- 0.93, p<0.0001) were found for all species. Therefore we used seed mass as a surrogate for seed set.

Results

Plant habit and floral biology: Gesneria species included in this study span three distinct floral designs: tubular and two types of bell-shaped flowers (campanulate and subcampanulate). *Gesneria citrina* has tubular, yellow flowers that are protogynous (Fig. 1). Floral characteristics are listed on table 1. Delayed self-pollination may or may not occur in *G. citrina* depending on the degree of herkogamy, which varies among plants (pers. obs.). *Gesneria cuneifolia* and *G. reticulata,* sister species according to our preliminary phylogenies, have tubular, red, flowers, that are also protogynous (Fig 1). Floral phenology, pistil length and nectar production differ between the two species (Table 1). In *G. cuneifolia*, delayed self-pollination can be achieved by the third day of flowering when stamens have elongated fully to the length of the stigma. In *G. reticulata*, self-pollination occurs during stamen elongation on the first day, or when stamen filaments bend to contact stigmas by the second or third day.

Gesneria pedunculosa has inflorescences that bear three to four white campanulate flowers with exserted reproductive organs (Fig. 1). The flowers are protandrous and schedules of anther dehiscence and nectar production are mostly nocturnal (Table 1). In second-day flowers mature stigmas grow to reach anther level and stigma receptivity may last through the third night. However self-pollination does not occur because stamens curl down below the stigmas after the first night of anthesis (also see bagging experiments below).

G. viridiflora subsp. *sintenisii* has subcampanulate flowers that have a constriction above the nectar chamber; corollas are green, and sometimes tinted with brown or violet markings (Fig. 1). Anthesis and nectar production start in the afternoon but anther dehiscence is nocturnal (Table 1). In this protogynous species, stigma receptivity starts with anthesis and may last through the second night, but self-pollination is rare because the stigma contacts only the back wall of the anthers (also see bagging experiments below).

Like all members of the tribe Gesnerieae, the Puerto Rican species have dehiscent capsules that contain hundreds to thousands of small seeds (pers. obs.). The seeds are most likely wind dispersed although in the case of *G. viridiflora* subsp. *sintenisii,* and perhaps other species, water dispersal is also possible. Fruit development takes two to three months.

Nectar measurements: Nectar volumes and production schedules differed among species (Table 1, Figure 2). Bell-shaped flowered species secreted significantly more nectar than tubular-flowered species ($F_{[3, 96]} = 76.1$, $p<0.0001$; Table 1). For tubular-flowered species, significant variation in nectar volume among time periods was detected ($F_{[3, 17]} = 37.9$, $p<0.0001$ for *G. citrina*, and $F_{[3, 30]} = 24.9$, $p=0.0004$ for *G. cuneifolia*). Nectar production in these species occurred mostly between 300 and 600 h, although in *G. cuneifolia* nectar accumulation continued throughout the morning (Fig. 2). *Gesneria* *reticulata* typically does not produce nectar, although we detected very small amounts of nectar (one to two µL per day) in a three out of 32 plants we tested (Table 1).

Nectar production in campanulate and subcampanulate-flowered *Gesneria* averaged between 60 and 62 µL (Table 1). Nectar volumes among time periods differed for both *G. pedunculosa* and *G. viridiflora* subsp. *sintenisii* ($F_{[3,15]} = 35.9$, $p<0.0001$ and $F_{[3,31]} = 83.1$, $p<0.0001$ respectively); the largest volume of nectar was secreted during the night time periods in both species (Tukey adjusted $p < 0.05$) followed by afternoon accumulation (between 1500 and 1800 h) and very little production during the morning hours (Fig. 2).

Sugar concentration values ranged from eight to 13 % with significant differences among species ($F_{[3,62]} = 11.8$, $P < 0.0001$). Sugar concentration was slightly but significantly lower for *G. viridiflora* subsp. *sintenisii* compared to *G. citrina* and *G. cuneifolia*, two tubular-flowered species (Tukey adjusted $p < 0.05$). *G. reticulata* had a significantly lower sugar concentration than all other species (Table 1).

Pollination systems: Hummingbirds were the exclusive pollinators of all three tubular-flowered *Gesneria* (i.e. *G. cuneifolia* and *G. citrina)* (Table 2). However, *G. reticulata* received only a single visit by *Chlorostilbon maugeaus*, the Puerto Rican Emerald, in 43 hours of observation. None of the observed hummingbird visitors exhibited territorial behaviour. Visitation frequencies to tubular flowers were in the range of one visit per flower every two or three days. All visits resulted in contact between anthers or stigma and the hummingbird's beak or forehead (Table 3). *Chlorostilbon maugeaus* also visit campanulate- and subcampanulate-flowered *Gesneria*. While these hummingbirds did not contact the flower's reproductive organs in *G. pedunculosa*, they often did in flowers of *G. viridiflora subsp. sintenisii* (Table 3).

Bananaquits (*Coereba flaveola*) are occasional nectar robbers of tubular-flowered *G. citrina* and *G. cuneifolia*; they pierce a hole at the base of the corolla and extract nectar without removing or depositing pollen. Bananaquits also visit bell-shaped flowers, feeding on nectar as nectar robbers (in *G. viridiflora* subsp. *sintenisii*), or by inserting their heads into the corolla and occasionally contacting the flower's reproductive organs (in *G. pedunculosa*).

Bats of the species *Monophyllus redmanii* are major pollinators of both *Gesneria pedunculosa* and *G. viridiflora* subsp. *sintenisii* (Table 2). Bat visits occur between 1900 h and 0600 h, lasting less than one second with peak visitation between 1900- 2300 and then again from 400- 600 h. Visitation rates for years with bats present ranged between two and four visits per flower per night (Table 2); no bats were observed in 2003.

Sphingid and noctuird moths also visit the flowers of bell-shaped flowered *Gesneria,* however moths rarely have the potential to be effective pollinators (Table 2). In *G. viridiflora* subsp. *sintenisii*, a number of noctuid moth species and perhaps other moth families visited some plants at high frequencies, particularly in 2003 and 2005. These moths crawl into the corolla moving in and out (by backing up) several times and visits may last several minutes.

Other visitors to *G. pedunculosa* included honey bees and flies (Muscidae and Syrphidae). Honeybees (*Apis mellifera)* are the most common visitors but we do not consider them legitimate pollinators. Honeybees remove pollen from anthers right before dehiscence, breaking down the connections among them and displacing anther filaments; this may affect future contact with legitimate pollinators. Honeybees do not contact stigmas while doing this or while foraging for nectar. Flies forage for leftover pollen;

occasionally they land on stigmas before moving to the anthers. *Gesneria pedunculosa* and *G. viridiflora* subsp. *sintenisii* are thus visited by varied assemblages of animal taxa, but the only visitors with potential to be important pollinators are bats in the former and bats and hummingbirds in the latter.

Self-compatibility and Autonomous selfing: All five species of *Gesneria* are self compatible; no significant differences in fruit set were found between hand-self pollinated and outcrossed flowers for any species (Table 4). There was no evidence of apomixis, none of the bagged emasculated flowers set seed. The levels of autonomous self-pollination varied among species (Table 4). The two species with campanulate and subcampanulate corollas had lower than 10% potential autonomous selfing rates. The three species with tubular flowers have relatively high potential autonomous selfing levels (25 % - 90% fruit set), however only for *G. cuneifolia* and *G. reticulata* was fruit set of bagged flowers the same as that of out-crossed flowers (Table 4). Seed mass did not differ among treatments for these two species either (*G. cuneifolia*, $F_{[1, 42]} = 0.97$, $P = 0.37$; *G. reticulata* $F_{[2, 48]} = 1.7$, $P = 1.9$). Seed mass of bagged flowers of *G .citrina* was significantly lower than seed mass of hand pollination treatments ($F_{[2,46]} = 7.61$, $P = 0.001$), indicating a lower potential for autonomous seed set in this species. *Gesneria reticulata* has the highest potential autonomous selfing rate as can be noted by the autonomous selfing index (AI=1). Considering the low hummingbird visitation, lack of nectar production, and high potential autogamy rate, this species can be considered predominantly selfing.

Discussion

Pollination systems: Plant-pollinator interactions in islands tend to be more generalized than their mainland counterparts as a consequence of the reduced diversity and abundance of animal pollinators that characterize insular ecosystems (Carlquist, 1974; Barrett, 1996; Olesen et al., 2002). However, highly specialized interactions have been documented in various islands, often associated with vertebrate pollination (e.g. Kodric-Brown *et al.,*1984, Temeles and Kress 2003, Micheneau *et al.,*2006). Here, we report specialization in two species of *Gesneria* from Puerto Rico, which are exclusively pollinated by one or two hummingbird species. Specialized pollination mutualisms are expected in the Caribbean islands where distance to the mainland is relatively short at both ends of the archipelagos, and animal dispersal by island hopping is possible. Hummingbirds in particular, have colonized remote islands such as Juan Fernandez, 667 km off the coast of Chile, where various species of plants with distinct hummingbird pollination syndromes are entirely dependant on these birds for outcross pollination (Bernardello *et al.,*2006).

Bats also have the ability of long-distance dispersal and are known pollinators of various island plants (e.g. Elmqvistt *et al.,*1994, Zusi and Hamas 2001). In Puerto Rico, bats were the most frequent and potentially most effective pollinators of *Gesneria pedunculosa*, although this species had a fairly diverse visitor assemblage (Table 3). Some floral visitors, such as bananaquits and flies, had low potential to transfer pollen (Table 3), while others did not contact stigmas during their visits (e.g. hummingbirds, hawkmoths, honey bees). These animals are most likely antagonists of the system that reduce the amounts of floral rewards available for legitimate pollinators (Thomson 2003).

Nevertheless, the presence of secondary floral visitors may be advantageous when legitimate pollinators become absent or scarce (Schemske and Horovitz, 1989; Thomson, 2003). For instance, in *Pilocereus royenii* -a Puerto Rican cactus species with flowers characteristic of bat pollination, only carpenter bees effected fruit set during a year of low densities of nectar feeding bats (Rivera-Marchand and Ackerman, 2006). Thus, temporal variation in pollinator assemblage might favour the maintenance of pollination generalization in species with distinct adaptations to particular pollinator guilds (Waser et al., 1996).

Temporal variation in bat visitation patterns was detected for subcampanulate-flowered *G. viridiflora* subsp. *sintenisii*; visitation rates ranged from one to four bat visits per flower per night (Table 3). *Gesneria viridiflora* subsp. *sintenisii* is a functionally generalized species pollinated primarily by bats and hummingbirds. Hummingbirds probably transfer little pollen during afternoon hours - before anther dehiscence time; however, early morning visits by hummingbirds may ensure pollination to flowers not visited during the night. Pollination by bats and hummingbirds has been reported for various plant species including *Abutilon* (Malvaceae) from Brazil (Buzato et al., 1994), *Marcgravia* (Marcgraviaceae) from the island of Dominica (Zusi and Hamas, 2001), *Burmeistera* (Lobeliaceae) from South America (Muchhala, 2006). However, in *Burmeistera* most species specialize on bat pollination (Muchhala, 2006), and tradeoffs for corolla shape appear to favour specialization to only one functional group, i.e., either bats or hummingbirds (Muchhala, 2007). The flowers of *G. viridiflora* subsp. *sintenisii* are intermediate in shape, but it is unclear whether or not subcampanulate flowers reflect selection imposed by two different pollinator guilds.

Floral rewards: Variation in nectar sugar concentration and amount of energy reward are known to influence attraction, foraging behaviour and energetics of different animal pollinators (Feinsinger, 1987; Baker and Baker, 1990). We found that the amount and schedule of nectar production was consistent with the schedules and energetic needs of the primary pollinators of *Gesneria*, however, there was little variation in sugar concentration (10 % - 13% ; Table 1), suggesting phylogenetic conservatism. High nectar volumes and low sugar concentration are characteristic of chiropterophilous flowers (Baker et al., 1998; Sanmartin-Gajardo and Sazima, 2005b; Tschapka and von Helversen, 2007) and these nectar traits have also been recently associated with pollination by generalist birds (Johnson and Nicholson, 2008). Overall nectar concentration for tubular-flowered Puerto Rican *Gesneria* is in the low range reported for *hummingbird-pollinated* plants (Pyke and Waser, 1981; Stiles and Freeman, 1993; Perrett et al., 2001; Nicolson and Fleming, 2003, Johnson and Nicholson, 2008), however, tubular-flowered *Gesneria* do not compensate by producing greater volumes of nectar. Low sugar concentration has been proposed as a strategy to deter bee visitation in ornithophilous flowers (Bolten and Feinsinger, 1978). However, this hypothesis is unlikely to explain the low nectar concentration in *Gesneria* because native bees are uncommon floral visitors of Antillean Gesneriaceae (our unpublished data).

Breeding systems: An alternative for plants that occur in environments where pollinators are scarce is to have breeding systems that provide reproductive assurance when natural pollination fails (Eckert et al., 2006; Fenster and Martén-Rodríguez, 2007). All Puerto Rican Gesnerieae are self-compatible but most are also dichogamous and only the three tubular-flowered species had relatively high selfing potentials (Table 3). One species, *G. reticulata*, is predominantly selfing and has evolved features associated with this mode of reproduction (i.e. lack of dichogamy, shortened pistils and little or no nectar production). Only one hummingbird visit was observed to *G. reticulata*, suggesting hummingbirds have learned to avoid the nectarless flowers despite their attractive appearance. Inference of the pollination system based exclusively on external morphology for *G. reticulata* would have incorrectly led to hummingbird pollination. Therefore, we emphasize the importance of conducting both pollination and breeding system studies simultaneously.

Conclusions: Puerto Rican *Gesneria* species display ornithophilous and chiropterophilous flowers and primary visitors generally correspond to those expected by floral syndromes. Our results provide evidence for highly specialized pollination systems in insular plant species; in one case specialization to bat pollination occurs despite the presence of a diverse visitor assemblage (in *G. pedunculosa*). Nevertheless, we also find evidence for pollination generalization; *G. viridiflora* subsp. *sintenisii* shows a mixed pollination system with hummingbirds and bats as potential major partners of the mutualism. Generalized pollination might buffer against demographic fluctuations of pollinators and/ or low floral visitation in tropical islands like Puerto Rico. Similarly, the presence of autonomous selfing in morphologically specialized *G. reticulata* suggests self-pollination is used as reproductive assurance in an environment where hummingbird visitation is low. This work highlights the usefulness of studying sets of closely related species to improve our understanding of the evolutionary and ecological aspects involved in the diversification of island plants.

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TABLE 1. Distribution, growth form and floral characteristics of five *Gesneria* species from Puerto Rico. Sample size is the number of plants examined for each species. In the nectar volume and concentration columns identical letters indicate the means are not significantly different at alpha 0.05 (Tukey adjustment).

Species	Distribution	Growth form	Floral design	Floral longevity No days	Breeding system	Anthesis time (Dehisence)	Nectar (µL) Mean/day [range]	% Sugar conc. Mean [range]
G. citrina	Central western karst	Scandent shrub	Tubular	4 -5 n=20	Protogynous	Before dawn (Second day)	12.8 [0-28] ^{a} n= 18	13. 3 [11-15] ^{a} n=17
G. cuneifolia	Central/ western karst	Roseate	Tubular	3-4 n=18	Protogynous	Before dawn (Second day)	5.2 [1-20] ^{a} n= 16	13.4 [11-15] ^{a} n= 12
G. reticulata	North east Rainforest	Roseate	Tubular	3-4 n=15	Adichogamous	Before dawn (First day)	0 [0-2] n=32	8.3 [8- 9] ^{b} n=3
G. pedunculosa	Central western karst	Shrub	Campanulate	2-3 n=20	Protandrous	1500-1700 h (after 1800)	60.3 [27-108] ^{b} n= 38	12.1 [11-14] ^{ac} n= 21
G. viridiflora subsp. *sintenisii*	North east Rainforest	Shrub	Subcampanulate	2-3 n=20	Protogynous	1500 - 1700 h (1700-1800 h)	62.0 [15 -128] ^{b} n=17	10.5 [7-15] $^{b\,c}$ n=16

TABLE 2. Floral visitors recorded for five *Gesneria* species from Puerto Rico between 2003 and 2007.

Species	Floral Design	Pollinators (common name)	Non-pollinating floral visitors (resource used)
G. citrina	Tubular yellow	*Anthracocorax viridis* (Green Mango) *Chlorostilbon maugaeus* (Puerto Rican Emerald)	Pterophoridae (plume moth larvae feeds on immature stamens) *Coereba flaveola* (nectar robber)
G. cuneifolia	Tubular red	*Chlorostilbon maugaeus*	*Coereba flaveola* (nectar robber)
G. reticulata	Tubular red	*Chlorostilbon maugaeus*	Not observed
G. pedunculosa	Campanulate	*Monophyllus redmanii* (Greater Antillean long-tongued bat) *Coereba flaveola* (Bananaquit)	*Chlorostilbon maugaeus* (nectar) Money bee (nectar, pollen) Sphingid moth (nectar)
G. viridiflora subsp. *sintenisii*	Subcampanulate	*Monophyllus redmanii* *Chlorostilbon maugaeus* Noctuid moths (various morphospecies)	*Coereba flaveola* (nectar robber) Small moths (Nectar)

TABLE 3. Frequency of visitation per flower per day and contact with reproductive organs by potential pollinators for five species of *Gesneria* from Puerto Rico studied during 2003 and 2005-2007. Number of visitors observed, number of study years and observation hours are indicated for each species.

Species	Pollinator	Mean no. of visits / flower per day [Range across years]	Mean percent contact with reproductive organs [Range]	no. of visitors	[no. of years] no. of hours
Gesneria citrina	Hummingbird	0.4 [0.3 – 0.5]	100	13	[3] 67
Gesneria cuneifolia	Hummingbird	0.5 [0.4 – 0.6]	100	6	[3] 41
Gesneria reticulata	Hummingbird	0.1 [0.0 - 0.2]	100	1	[3] 43
Gesneria pedunculosa	Bat	3.9 [3.7 – 4.2]	100	39	[3] 91
	Bananaquit	1.0 [0.7 – 1.6]	41 [29 – 56]	7	
	Pollen collectors	0.6 [0.0 – 0.9]	68 [60 – 75]	6	
Gesneria viridiflora	Bat	2.5 [1.3 – 3.6]	100	6	[3] 84
subsp. *sintenisii*	Hummingbird	3.0 [2.5 – 3.7]	61 [57 – 65]	12	
	Moth	3.3 [3.0 – 3.6]	7 [0 – 14]	16	

TABLE 4. Tests for self-compatibility and autonomous selfing for five *Gesneria* species from Puerto Rico performed in January 2006. Least square means (± SEM) for fruit set are reported for each pollination treatment. Identical letters indicate no significant differences among means at the 0.05 alpha (Tukey adjustment).

Species	Hand-outcross	Hand-self	Bagged	F values	p	AI (1)
G. citrina	64 ± 5.8 **a**	56 ± 5.0 **a**	24 ± 6.4 **b**	$F_{(2,47)} = 8.7$	< 0.001	0.38
G. reticulata	89 ± 5.2 **a**	92 ± 5.4 **a**	90 ± 5.3 **a**	$F_{(2,48)} = 0.08$	0.92	1.00
G. cuneifolia	77 ± 7.1 **a**	82 ± 6.7 **a**	68 ± 9.3 **a**	$F_{(2,42)} = 0.75$	0.48	0.88
G. pedunculosa	60 ± 4.0 **a**	55 ± 3.3 **a**	1.2 ± 4.5 **(2)**	$F_{(1,46)} = 1.04$	0.32	0.02
G. viridiflora	64 ± 5.1 **a**	59 ± 5.0 **a**	7.6 ± 2.8 **b**	$F_{(2,51)} = 24.5$	< 0.001	0.09

(1) Autonomous selfing index = Fruit set of bagged plants / Fruit set of hand-outcrossed

(2) Bagged treatment not statistically compared because most values were zeros.

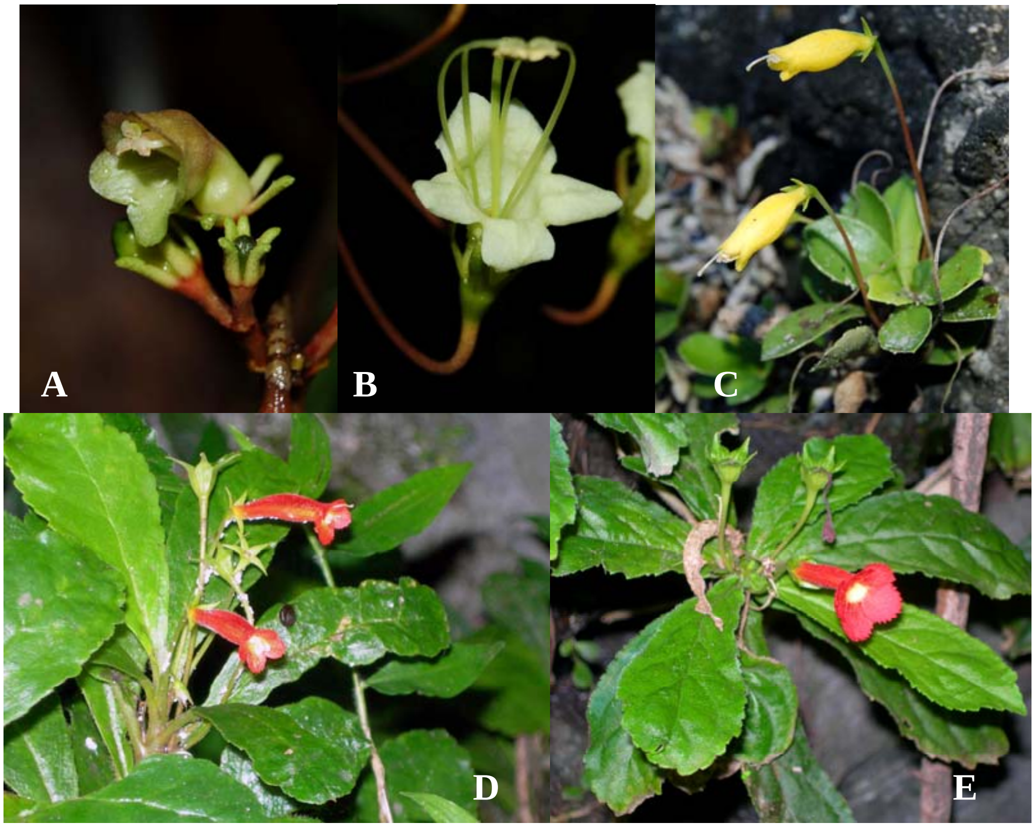

FIGURE 1. Five species of *Gesneria* from Puerto Rico: (A) subcampanulate *Gesneria viridiflora* subsp. *sintenisii*, (B) campanulate *Gesneria pedunculosa*, (C) tubular *Gesneria citrina*, (D), tubular *Gesneria cuneifolia*, (E) tubular *Gesneria reticulata*.

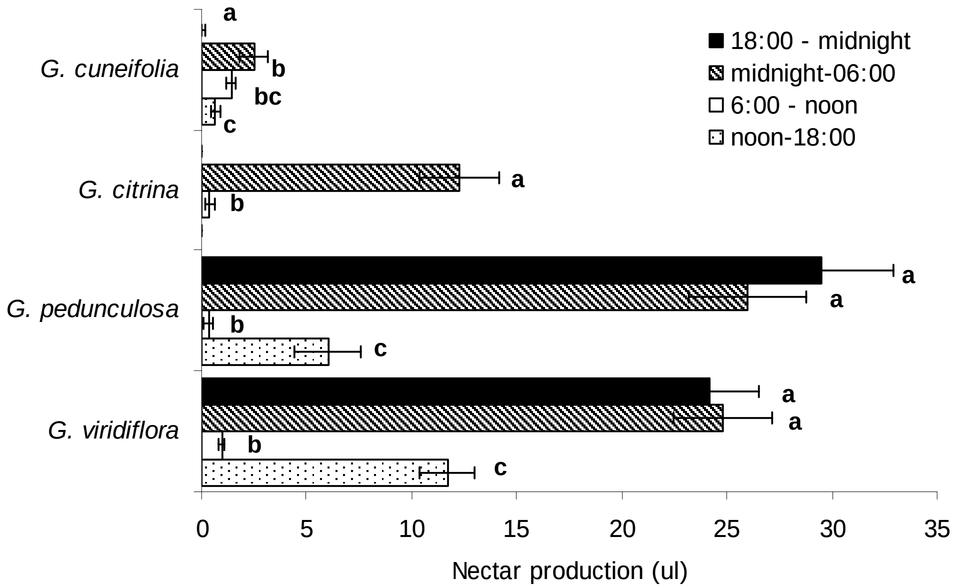

FIGURE 2. Average nectar production per six hour period recorded in January 2007 for four species of *Gesneria* endemic to Puerto Rico. Bars represent ± 1 standard error. Identical letters indicate means among time periods within species are not significantly different. In *G. cuneifolia* and *G. citrina*, nectar production starts after 0300 h.

CHAPTER 2

An evaluation of pollination syndromes in Antillean Gesneriaceae: evidence for bat, hummingbird and generalized flowers.

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Abstract

Current views about the predominance of generalization of pollination systems have stimulated controversy concerning the validity of pollination syndromes. In order to assess the extent to which floral characters reflect selection by the most important pollinators we evaluated pollination syndromes in a florally diverse plant group, the tribe Gesnerieae, a monophyletic plant radiation from the Antillean islands. The study species include representatives of three groups of floral phenotypes, two of which chiefly correspond to ornithophilous and chiropterophilous syndromes. The third group includes subcampanulate flowers (characterized by a corolla constriction above the nectar chamber) with combinations of traits not fitting classic pollination syndromes. Pollination systems were characterized for 19 Gesnerieae species in five Antillean islands between 2003 and 2007 and supplemented with observations of four outgroup species from Costa Rica. Pollinator visitation and frequency of contact with anthers or stigmas were used to calculate an index of pollinator importance. Eleven floral traits including morphology, phenology and rewards were used to assess clustering patterns in phenotype space. Multidimensional scaling analysis of floral traits resulted in two clusters comprising: (1) tubular, red to yellow-flowered species with diurnal anthesis, 2) bell-shaped-flowered species; two groups of floral phenotypes were evident within the latter cluster, campanulate nocturnal and subcampanulate flowers. Correlations between pollinator importance values and floral axes revealed strong associations with the expected pollinators, hummingbirds for tubular flowers, and bats for campanulate flowers; subcampanulate-flowered species had generalized pollination systems including bats, hummingbirds and insects. Discriminant analysis of the multivariate set of floral traits correctly classified 19 out of 23 species into the predicted pollination categories. This study provides support for classic hummingbird and bat pollination syndromes, demonstrating the importance of pollinator-mediated selection in the floral diversification of Antillean Gesnerieae. However, there was evidence for generalized pollination systems in species characterized by a unique morphological trait (corolla constriction), but with variable combinations of other floral traits. These findings suggests that floral phenotypes might also evolve under selection by various functional groups of pollinators, and underscores the importance of considering the presence and effectiveness of all floral visitors in pollination studies.

Key words: Antilles, bat pollination, Costa Rica, generalization, Gesneriaceae, hummingbird pollination, islands, pollination syndromes, specialization.

Introduction

Closely related plant species often display great variation in flower form and function. Darwin (1862) proposed that the evolution of this floral diversity reflected pollinator-mediated selection. Patterns of convergence of floral phenotypes across the angiosperms provide strong support for Darwin's paradigm and suggest that suites of floral characteristics have evolved in association with particular groups of pollinators. These floral phenotypes are known as 'pollination syndromes' (Faegri & van der Pjil 1978), and they comprise morphological as well as biochemical (e.g. composition of attractants and rewards, Baker & Baker 1990) and phenological traits (e.g. patterns of anther dehiscence, Castellanos *et al.* 2006). For example, large bell-shaped flowers that produce large quantities of dilute nectar and shed pollen at night tend to be associated with bat pollination, while tubular red flowers with diurnal schedules are commonly associated with pollination by birds. Syndromes therefore imply that flowers have become specialized for pollination by specific groups of floral visitors, i.e., traits have evolved to increase pollen transfer by the most effective visitors and to deter antagonistic visitors (Stebbins 1970; Faegri & van der Pjil 1978; Fenster *et al.* 2004).

During the past decade, however, the notion that pollination specialization underlies the observed patterns of floral convergence has been debated (Waser *et al.* 1996, Fenster *et al.* 2004). Community and taxon surveys predominantly from temperate regions reveal that many flowers have generalized visitation patterns (e.g. Robertson 1928; Lindsey 1984; Herrera 1996, Olesen *et al.* 2007), pollinator communities vary in time and space (e.g. Herrera 1995; Fenster & Dudash 2001; Horovitz & Schemske 2002), and animals often use floral resources from different plant species (e.g. Herrera 1996). Furthermore, syndromes do not predict all floral visitors, and flowers that conform to particular syndromes are sometimes pollinated by animals that do not fit the expectations (Ollerton *et al.* 2007). These observations have led some authors to question the validity of the pollination syndrome concept (Waser *et al.* 1996; Ollerton *et al.* 2007). While syndromes were not originally meant to be used as substitutes for field observations, there is a valid concern regarding the use of floral traits as predictors of the pollinators, particularly when biased or no field data have been collected (Feinsinger 1987; Waser *et al.* 1996). Obtaining impartial characterizations of pollinators at the level of communities or higher order plant taxa, particularly from understudied tropical regions, is critical to solving the apparent disagreement between observed evolutionary patterns of floral specialization and the patterns suggested by field ecology (Johnson & Steiner 2000). Furthermore, despite the clear difficulties involved in obtaining direct measures of pollinator efficiency, an attempt should be made at distinguishing between potential pollinators and non-pollinating floral visitors. This approach should lead us to a more comprehensive understanding of the different selective agents that have influenced the great floral diversification observed in some plant taxonomic groups.

Pollination studies of closely related insular species could provide important insights into the selective pressures that underlie patterns of floral convergence. We studied the Antillean tribe Gesnerieae to determine to what extent floral phenotypes defined by morphology, phenology and rewards are explained by the pollinators. This group of plants provides an excellent study system for various reasons. First, the tribe Gesnerieae is a monophyletic radiation from the Antilles that consists of 73 species encompassing considerable floral diversity (Skog 1976; Smith 1996; Zimmer *et al.* 2002). Second, this floral diversity comprises floral phenotypes that have been traditionally associated with hummingbird and bat pollination, but previous to our studies, no field data were available to support these predictions. Furthermore, despite the great floral diversity displayed by

Neotropical Gesneriaceae, only a limited number of studies have documented pollinators in the field (e.g. Carlson 2008, Lara & Ornelas 2002, Sazima *et al.* 1996, Podolsky 1992), and only for the tribe Sinningieae in Brazil has there been a systematic assessment of pollinators in a group of closely related species (Sanmartin-Gajardo & Sazima 2004, 2005a,b). Third, oceanic archipelagos provide unique conditions of natural selection and opportunities for evolutionary change that may differ from mainland regions. And last, our preliminary molecular and morphological phylogenies indicate at least five independent origins of bell-shaped corollas that differ from the tubular corollas of the ancestral phenotype; this suggests pollinators played a significant role in the floral diversification of the clade. We included four species from three additional tribes of the family Gesneriaceae from Costa Rica to obtain phylogenetically independent evidence from mainland taxa. The selected species fall into three general classes of floral phenotypes corresponding to ornithophilous and chiropterophilous syndromes, and a class of more variable phenotypes that do not clearly match classic syndrome predictions (Fig. 1).

For this study, we specifically address the following questions: (1) what are the pollination systems of Gesneriaceae species representative of the different floral phenotypes?; (2) when floral traits are used to search for patterns in multivariate space, is there evidence for discontinuous associations of species corresponding to traditional pollination syndromes?; (3) which floral traits contribute most to distinguishing the floral associations defined in multivariate space?; and (4) are pollination syndromes good predictors of the floral visitors for Antillean Gesneriaceae? We evaluate the predictions that tubular flowers in the Gesnerieae are primarily pollinated by hummingbirds, and bell-shaped (campanulate and subcampanulate) nocturnal flowers are primarily pollinated by bats. We also provide the first descriptions of pollinators for *Rhytidophyllum* species with mixed floral traits not fitting classic pollination syndrome categories.

Methods

Study sites: Pollinator observations and floral biology studies were conducted in Costa Rica (February-March 2007), Cuba (September 2007- February 2008), the Dominican Republic (June-August 2004-2007), Jamaica (January 2004) and St. Lucia (June 2003). *Rhytidophyllum minus* was observed at Castillo San Pedro de la Roca located on coastal limestone cliffs, south of the city of Santiago, in western Cuba. In the Dominican Republic, plants were observed at various sites of Cordillera Central, Cordillera Septentrional, Parque Nacional Sierra de Bahoruco and Sierra Neiba. All of these sites are located in mountain regions between 300 and 2000 m. Most species occur in limestone soils but they occupy a diversity of habitats including pine forests, moist and cloud forests, and roadsides. In Jamaica, *Gesneria calycosa* plants were observed in the forest surrounding Windsor Biological station in the NW side of the island (a.k.a. cockpit country) and *Pheidonocarpa corymbosa* at Cane River Falls in the foothills of the Blue Mountains. In St. Lucia, observations of *G. ventricosa* were conducted in Edmund Forest along the road to En Vasseux Waterfall, 500 m. Specific localities and geographic coordinates for the study sites for each species are listed in Appendix I.

To obtain phylogenetically independent samples, we also observed four species of Gesneriaceae from three different tribes (Beslerieae, Gloxinieae and Episcieae, according to Zimmer *et al.* 2002) that occur in Costa Rica. Observations for these species were conducted in the forest of the Biological Station in Monteverde (for *Besleria solanoides*) and in the rainforest of San Gerardo Biological Station (for *Capanea grandiflora, Columnea consanguinea* and *Columnea quercetii*); these sites are located on the western and eastern slopes of the Tilarán Mountain range, respectively. We also use data from a detailed study of the pollination biology of five Puerto Rican *Gesneria* performed in January and March 2003-2007 in two regions of the island (Martén-Rodríguez & Fenster 2008).

Pollinator visitation and importance: To document pollinator visitation we conducted field observations on 23 species of Gesneriaceae for a total of 602 hours. Detailed descriptions of the methodology and floral biology of five Puerto Rican species of *Gesneria* are reported elsewhere (Martén-Rodríguez & Fenster 2008); thus, we only briefly describe the methods for pollinator observations here. The total number of individuals observed per species ranged from eight to sixty, depending on the population size and density of each species. The number of study years varied from one to three, but for each floral phenotype at least three species were observed for more than one year. For most species we made both direct observations and observations with video cameras (SONY Handycam DCR-HC42 and DCR-TRV350); the observer or the camera stood two to five metres from the focal plant and recorded the time of visitation, type of visitor (e.g. bird species, bat, moth, and diurnal insect order or family), contact with the flower's reproductive organs, and the number of flowers visited. Both diurnal and nocturnal observations were conducted for most species. For species with nocturnal and diurnal visitors, approximately half to three quarters of the time reported was dedicated to nocturnal observation. The larger time effort put into night observations was necessary to compensate for the limited number of flowers that video cameras could be focused on at night (one to four), as opposed to the ability to conduct direct observation on patches of flowers during the day.

We classified pollinators into "functional groups", defined on the basis of taxonomic affinity and similarity in feeding behaviour. Functional groups are expected to represent sets of animal taxa that exert similar selective pressures on floral traits, because they share similar feeding behaviours, physiology and morphology (Fenster *et al.* 2004). For this study the taxonomic classes of floral visitors differed primarily in feeding schedule (active at flowers during day or night), reward sought (pollen or nectar) and behaviour while feeding (e.g. hovering vs. perching). Thus, the functional groups of pollinators that visited Gesnerieae species include hummingbirds, bats and diurnal insects (small bees and flies that visited flowers primarily for pollen). For each year, pollinator visitation rates by each pollinator functional group were calculated as the number of visits per flower per hour; this rate was multiplied by 12 to obtain visitation frequencies per day or night, according to the schedule of the pollinator. At the latitudes where the study was conducted, daylight hours range between 12 and 13; thus, for the sake of consistency, we calculated visitation for 12-hour days.

To distinguish non-pollinating floral visitors from animals that have the ability to transfer pollen we carefully observed visitor behaviour and frequency of contact with the reproductive organs. Whenever possible we observed virgin flowers and checked them after a visit to determine whether pollen had been removed from anthers or deposited onto stigmas. However, since these data were collected only for a subset of the flowers, we quantified efficiency as the number of times the visitor contacted stigmas or anthers divided by the total number of visits (Armbruster & Herzig 1984). We recognize that contact is an approximate measure of efficiency but due to the logistical difficulties of obtaining pollen removal and deposition data for a large group of species, we consider this approach provides a better characterization of the pollination system than a simple list of floral visitors. Pollinator importance values for each group of visitors were calculated as the product of visitation and efficiency. To obtain a comparable index of pollinator importance we standardized each value, dividing it by the sum of importance values across all functional groups of pollinators. Therefore, pollinator importance indices range from 0-1.We report mean pollinator visitation and range across years for species observed for more than one year. Importance values obtained from one year of sampling, particularly those of bats and infrequent insect visitors, may not be accurate estimators. However, we have a representative sample of species (including the principal floral phenotypes) that were observed for many hours in multiple years. Since these results are mostly consistent across the data set, we considered it appropriate to include the understudied species. We excluded visitors that were never observed contacting the reproductive organs or carrying pollen (e.g. grasshoppers, beetles) and the introduced honeybee *Apis mellifera*, since it is unlikely this species has been long enough in the New World (a few hundred years) to be responsible for evolutionary changes underlying floral diversification of the tribe Gesnerieae.

Measurements of floral traits: To characterize floral phenotypes we measured 11 floral characters from two to three flowers of 7-23 individuals per species. Flowers were collected from all plants available when population densities were low (< 20 individuals); otherwise, flowers were collected from a sample of the population. Flower measurements of fresh flowers included: 1. Corolla length, the shortest length of the corolla tube; 2. Pistil exertion, measured in pistillate-phased flowers as the difference between pistil length and corolla length; 3. Diameter of the corolla opening; 4. Corolla constriction, coded as present or absent; 5. Corolla curvature, taken with a protractor for curvature of the dorsal side of the corolla tube; 6. Nectar concentration (see below); 7. Symmetry, coded as (0) subactinomorphic (reproductive organs not symmetrically positioned, otherwise actinomorphic) or (1) zygomorphic; 8. timing of anther dehiscence, coded as (0) nocturnal (18.00- 06.00), (2) diurnal (06.00-18.00), or (1) both; 9. Timing of nectar production, same coding as trait eight; 10. Colour, coded as (1) green, (2) yellow, (3) orange or (4) red; 11. Spots: coded for (0) presence or (1) absence of dark red or brown markings on the inside of the corolla. Measurements of length, width and pistil exertion were taken with calipers and rounded to the nearest 0.1 mm. Floral measurements for most species were taken by one person (S. Martén-Rodríguez), except for measurements for *R. minus*, which were taken by A. Almarales-Castro; all measurements are listed in Appendix II.

To document the timing of anthesis and nectar production, two species of each floral phenotype were selected (nocturnal campanulate and subcampanulate: *Gesneria fruticosa, G. quisqueyana*; tubular: *G. pedicellaris, Rhytidophyllum asperum*; subcampanulate mixed traits: *R. leucomallon*, *R. vernicosum*). We also used data for five Puerto Rican *Gesneria* previously studied (Martén-Rodríguez & Fenster 2008). Flower buds of one to two flowers per plant, in five to 14 plants per species were bagged and checked every three hours for a continuous 24-hour period starting at 15.00; the earliest time at which anther slits were noticed open was recorded. Nectar was extracted from bagged first-day flowers using capillary tubes or with a 50 μl Hamilton Syringe

(Hamilton, NV, USA), and sugar concentration was measured with a hand-held refractometer (Sugar/Brix Refractometer, 0–32% w/ATC, Sper Scientific, Scottsdale, AZ, USA). For the remaining species, plants were checked at least four times over the course of 24 h, such that we could tell whether pollen shedding occurred at night (18.00-06.00) or day (06.00-18.00). Nectar concentration on these plants was measured in flowers that were not previously bagged. Nectar production coded as nocturnal started as early as 15.00 and generally stopped by 07.00; nectar production coded as diurnal started as early as 04.00 and stopped at different times of the day, depending on the species. For anther dehiscence, nocturnal schedules were generally from 18.00-20.00 while diurnal anther dehiscence started as early as 05.00. Nectar volume was not included because this measurement required bagging flowers and sample sizes were insufficient to obtain reliable measurements for almost half of the species. In order to identify scent production, first-day flowers of at least two species of each floral phenotype (nine species total) were left in glass containers for two to three hours and then checked by smelling them. No perceivable scent was detected for any of these species; thus, this trait was not included in the analysis.

Statistical Analyses: All analyses were performed in SAS version 9.1.3 (SAS Institute, 2004). We used multidimensional scaling to examine patterns of association among species with floral characters traditionally linked with pollination syndromes. Eleven floral characters described above were used to calculate dissimilarity matrices using the DISTANCE procedure with method=dgower specified to calculate distances based on Gower’s coefficient; this coefficient allows the use of nominal and different kinds of quantitative variables (Gower 1971). The MDS procedure using the ordinal level option was used to indicate non-metric ordinations. Two ordinations using floral characters were performed: one that included all species and one that excluded species with tubular flowers. The latter analysis was conducted in order to evaluate floral characters that might help discriminate between bat-pollinated and generalist species. Multidimensional scaling by pollinator visitation and importance values was also performed to compare the grouping patterns produced by floral characters with the groupings suggested by the pollinators. The patterns using visitation data were the same as those using importance values; therefore, we report only the latter below.

The sample of species used in the floral ordination was not phylogenetically corrected; therefore, some similarities among species may reflect common ancestry. Aware of this problem, we attempted to ensure a higher level of independence by including, for both chiropterophilous and ornithophilous flowers, species from at least two clades within the Antillean tribe Gesnerieae (Martén-Rodríguez *et al.*, unpublished) and four Costa Rican species from three additional tribes (Zimmer *et al.* 2002). Thus, we can be confident that the results reported below reflect more than taxonomic affinities.

To evaluate the association between floral characters or pollinator importance with the first two dimensions of the ordination, we conducted Spearman rank correlations. Correlation coefficients are reported significant at the p=0.05 level after sequential Bonferroni correction to adjust for multiple comparisons. Likewise, Spearman rank correlations were used to assess the degree of association among floral characters.

To evaluate the predictability of pollination syndromes in Antillean Gesneriaceae, we conducted non-parametric discriminant analysis using the first two dimensions of the floral ordination (all species included) to represent the suite of floral traits. We used the

DISCRIM procedure in SAS, specifying the ‘kernel normal’ option to allow for a non-linear discriminant search. We made no assumptions about the underlying multivariate distribution and used a non-pooled covariance matrix. *A priori* groupings were based on the observed pollination systems (i.e. hummingbird specialists, bat specialists and generalists). To classify species as generalist or specialist we used our pollinator importance index, which combines visitation frequency and frequency of contact with the reproductive organs. Fenster *et al.* (2004) used a cut-off of a 75% visitation frequency for the most abundant pollinator to determine their categories of specialization and generalization. Here, we utilized a natural break in the data near that cut-off point and classified species as generalists if the importance index of the most important pollinator was 76 % or below, and as specialists if the index was 77 % or higher. The lowest most important pollinator index for species classified as specialists was 84% (for *G. pedunculosa*).

Results

Pollinator visitation and importance: A list of the floral visitors observed in all Costa Rican and Antillean Gesneriaceae is provided in Appendix III. Pollinators of tubular-flowered species were almost exclusively hummingbirds, usually one or two hummingbird species. Visitation rates by hummingbirds to Gesneriaceae species from the Antillean islands ranged from one visit per flower every three days to two visits per flower per day; visitation rates to Costa Rican Gesneriaceae ranged between three and six visits per flower per day (Table 1). Hummingbird pollinator importance values for tubular-flowered species ranged between 0.96-1.00; other visitors included Halictid bees and butterflies, with low importance values (0.03-0.04). We found little temporal variation in visitation rates and pollinator importance values for species that were observed in multiple years (Table 1).

Gesneriaceae species with campanulate green or white flowers were primarily pollinated by bats; birds and insect visitors, when present, had low importance values (Table 1). Visitation rates by bats ranged between one and four visits per flower per night and importance values between 0.80-1.00 (Table 1). As reported in a previous study, subcampanulate *G. viridiflora* subsp. *sintenisii* from Puerto Rico was pollinated both by bats and hummingbirds; pollen is available for pollen transfer by hummingbirds in late afternoon (second-day flowers), and at dawn (unvisited third-day flowers); therefore we consider this species an ecological generalist despite its mostly nocturnal pollination syndrome (Martén-Rodríguez & Fenster 2008). In contrast, *G. quisqueyana* from the Dominican Republic and sister to *G. viridiflora* subsp. *sintenisii*, restricts access to diurnal visitors by an active exclusion mechanism. The flowers of *G. quisqueyana* are protogynous; however, unlike its bat-pollinated relative, which has mid-afternoon anthesis, flowers of *G. quisqueyana* open between 19.00 and 20.00 and the pistillate phase lasts only one night. Corollas close up completely the next morning between 06.00 and 07.00 h and open the second and last night in male phase; receptive stigmas are not exposed during the day.

Generalized pollination systems were characteristic of *Rhytidophyllum* species with subcampanulate corollas and mixed combinations of other floral traits. The two-day protogynous flowers were visited by different sets of animal taxa, including bats, hummingbirds, moths and small diurnal insects (Halictid bees and flies). All these animals contacted stigmas and anthers at least occasionally, but differences in efficiency among visitors may be considerable. Bat and hummingbird visits often result in pollen removal and deposition (checked on virgin flowers after one visit), and large pollen loads deposited on foreheads or bills. In contrast, most insect visitors carry little pollen. Overall visitation to generalist flowers ranged from 3 to 26 visits per flower per day (Table 1).

Ordinations by floral traits and pollinator importance: Two distinct clusters separate along dimension 1 of the floral ordination, corresponding to tubular and bell-shaped flowers (both campanulate and subcampanulate) (Fig. 2). The cluster of tubular flowers includes species from various clades (Zimmer *et al.* 2002, Martén-Rodríguez *et al.*, unpublished), and are all strictly hummingbird-pollinated. Within the cluster of species with bell-shaped flowers, two subgroups can be distinguished, one associated with bat pollination (above the zero value of dimension 2), and the other associated with generalized pollination (mostly below the zero value).

Most floral characters were highly correlated with the first dimension of the ordination plot. The correlations indicate that, moving towards the left side of the plot along dimension 1, flowers have wider corollas, some constriction above the nectar chamber, lower nectar concentration, nocturnal schedules of nectar production and anther dehiscence, colours towards the yellow/green part of the spectrum, and the presence of dark red spots (Fig 2). In contrast, moving to the right side along dimension 1, the trend is for tubular corollas with solid bright colours, greater nectar concentration, and diurnal nectar production and anther dehiscence (i.e. hummingbird pollination syndrome). For the colour trait, which was coded as a multi-state character, the coding was set to reflect the colour spectrum; therefore, moving to the right along the dimension 1 indicates more orange and red corollas.

The ordination conducted excluding tubular-flowered species shows a stronger separation of the two subgroups of bell-shaped flowers; however, two oddities are evident: *Rhytidophyllum minus* (RM) appears clustered within the generalists but only hummingbirds were observed as native pollinators. Given its nocturnal schedule of nectar production and anther dehiscence, we cannot rule out the possibility of bat pollination until observations in multiple seasons are conducted. The second inconsistent case is *G. quisqueyana* (GQ), a strict bat specialist that was placed within the generalists cluster. This species restricts diurnal visitors by closing flowers during the day (see above description). Thus, although the floral morphology would allow a wider range of visitors, the floral phenology filters out diurnal visitors.

With tubular-flowered species excluded from the ordination, the corolla constriction became the single most important trait separating the two subgroups of bell-shaped flowers (associated with generalized and bat pollination) (Fig. 3). Other high correlations (significant before Bonferroni correction) included: pistil exertion, corolla curvature, and colour. Thus, moving to the right along dimension 1 (associated with specialized bat pollination), pistils tend to be more exerted, corollas less curved, light green or white, and not constricted above the nectar chamber.

Pollinators also separated Gesneriaceae species into clusters corresponding to ornithophilous and chiropterophilous flowers in the ordination using pollinator importance values (Fig. 4). However, in contrast with the clustering defined by floral traits (Fig.1), species with subcampanulate flowers appeared scattered throughout the plot, reflecting the variability in pollinator importance values and pollinator assemblages

(Fig. 4). The only trait that correlated with dimension 1 was colour, indicating red colours present in most species visited by hummingbirds, both specialists and generalists.

Correlations among floral traits revealed 11 significant associations (Table 2). These indicate flowers with wide corollas tend to have nocturnal schedules, green to white colours and dark red or brown spots, while flowers with narrow corollas tend to have diurnal schedules and solid bright red or orange colours. These associations reflect the suites of floral characters associated with classic bat and hummingbird pollination syndromes, respectively. The presence of a corolla constriction that makes subcampanulate corollas, was associated with nocturnal schedules of nectar production and anther dehiscence. As a general rule, this subcampanulate floral phenotype indicates generalized pollination systems in the tribe Gesnerieae.

Evaluation of pollination syndromes: Discriminant analysis was used to evaluate the ability of suites of floral traits to predict the pollination system; the three *a priori* designated pollination system categories were based on our field observations: hummingbird, bat and generalist. When cross-validation was used to evaluate the ability of the model to classify species into expected pollination systems, floral traits were able to predict hummingbird pollination 12 out of 13 times; *Rhytidophyllum minus* was classified as a generalist (Table 3). For the bat pollination category, one species out of five was misclassified (*G. quisqueyana* was classified as a generalist), and for the generalists two out of five species were misclassified, one into the hummingbird (*R. leucomallon*) and one into the bat (*G. viridiflora* subsp. *Sintenisii*) pollination categories (Table 3).

Discussion

The validity of the pollination syndrome concept has been recently called into question based on an argument derived primarily from the observed widespread generalization of pollination systems in temperate regions (Waser *et al.* 1996; Ollerton *et al.* 2007), although some recent community-level studies suggest generalized pollination systems may be equally common in the tropics (Ollerton & Cranmer 2002). This study evaluated the correspondence between pollination ecology and patterns of floral diversity in the Antillean monophyletic tribe Gesnerieae. To obtain a better idea of the animals that could be agents of selection on floral characters, we made an effort to distinguish floral visitors that have the ability to transfer pollen, from non-pollinating visitors. We also attempted to reduce underestimating the number of potential pollinators by surveying a subset of the species for various years and at various sites. Our study provides evidence for both extreme ecological specialization and generalization within a group of Neotropical Gesneriaceae, and demonstrates that the occurrence of ecological generalization (visits by many species) has not precluded the evolution or maintenance of suites of floral traits that coincide with established pollination syndromes (Faegri & van der Pjil 1978).

The patterns for bat and hummingbird pollination syndromes were the same for Antillean and more distantly related mainland Gesneriaceae; no generalists from the mainland were identified in this study. Gesnerieae flowers show adaptations to bat pollination that correspond to traits that have been described in over 700 bat-pollinated species of tropical and subtropical plants (Tschapka & Dressler 2002), including other members of the family Gesneriaceae (e.g. floral morphologies that facilitate access to nectar, open corollas to facilitate detection by echolocation, exposed floral displays to enhance accessibility; San Martin-Gajardo & Sazima 2005b). Our results also support the existence of a distinct hummingbird pollination syndrome in the Gesneriaceae (tubular, red or yellow diurnal flowers with dilute nectar), one of the most widespread and accepted patterns of floral convergence (e.g. Sakai *et al.* 1999; Kay & Schemske 2003; Hargreaves *et al.* 2004; Sanmartin-Gajardo & Sazima 2005a; Wilson *et al.* 2006; Whittall & Hodges 2007). However, an intriguing finding was the occurrence of high hummingbird visitation to flowers that obviously do not correspond to the ornithophilous syndrome (i.e. visitation to bell-shaped, green/light yellow flowers).

Although hummingbird visitation to 'non-ornithophilous' flowers had been previously observed (e.g. Feinsinger 1976; Stiles 1976), the significantly greater visitation to the green flowers of *Gesneria* was unexpected. In a sample of Gesnerieae from Puerto Rico and the Dominican Republic we found that the average nectar volume was greater for bell-shaped-flowered species (range 60-82 μL, n= 4 species), than in tubular-flowered ones (range 5-16 μL, n=5 species; Martén-Rodríguez & Fenster 2008 and unpublished data). Nectar volume, a trait that clearly separates bat- from hummingbird-pollinated species was not included in this study due to the small number of species for which accurate estimates were obtained. However, it appears that hummingbirds are attracted to green bell-shaped-flowered *Gesneria* species due to their higher nectar content. Other floral traits associated with the ornithophilous syndrome may serve to enhance efficiency of pollen transfer (tubular corollas; e.g. Castellanos *et al.* 2004), or signal the presence of a common food source (corolla colour), but are not the primary attractants for the birds (Stiles 1976).

The lack of fidelity by hummingbirds to species with tubular corollas explains the existence of generalized pollination systems. Gesnerieae species with subcampanulate corollas and mixed floral traits had nocturnal (bats and moths) and diurnal (hummingbirds and flies) visitors potentially contributing to fruit set. Floral traits that coincide with bat pollination are: nocturnal schedules of nectar production and anther dehiscence, abundant dilute nectar, and light yellow-green corollas in most species. Although some of these traits also correspond with known adaptations to moth pollination, moths do not always contact stigmas or anthers. In contrast, some traits appear to be driven by selection to enhance hummingbird pollination. These traits vary among generalist Gesnerieae, but they include narrower bell-shaped corollas, yellow colours with variable amounts of bright red markings, and diurnal as well as nocturnal nectar production and anther dehiscence (in *Rhytidophyllum vernicosum*). No floral adaptations for the rare and inconsistent diurnal insect visitors were detected, as indicated by the lack of correlation between these visitors and floral traits (Figs. 2-4). Species with intermediate phenotypes between ornithophily and chiropterophily have been described in at least two other plant families: the Lobeliaceae (e.g. *Syphocampylus sulfureus,* Sazima *et al.* 1994) and the Malvaceae (e.g. *Abutilon,* Buzato *et al.* 1994). In both cases, floral traits have been interpreted as transitional phenotypes along an evolutionary pathway to bat pollination. There are other instances where bat- and hummingbird-pollinated species occur within the same genus but no intermediate phenotypes are found in nature, reflecting trade-offs in corolla shape imposed by bats and hummingbirds (Muchhala 2007).

In Gesnerieae, some traits display character states that appear to reflect selection by two different functional groups of pollinators, such as nocturnal and diurnal schedules of nectar production and anther dehiscence, and colour variation in some species. Whether these characters represent a transitional stage or an equilibrium point maintained by divergent selective pressures exerted by nocturnal and diurnal pollinators is not clear. However, these traits do not consistently explain the phenotypic clustering of species with generalized pollination systems. The single trait that distinguished generalists from their bat-pollinated relatives was the presence of a corolla constriction located right above the nectar chamber (see Fig. 1). Wolfe and Stiles (1989) proposed that corolla constrictions in hummingbird-pollinated flowers were part of an adaptive 'fail-safe' mechanism that enticed visitation by secondary pollinators, when the primary hummingbird specialists were absent. We hypothesize the corolla constriction in *Gesneria* and *Rhytidophyllum* facilitates nectar access to bats, while increasing the effectiveness of hummingbird pollination, thus promoting a dual pollination strategy in flowers predominantly adapted for bat pollination.

In the generalist species of *Gesneria* and *Rhytidophyllum*, hummingbird visits occur mostly in the late afternoon and early morning, which could be interpreted as thieving of early or leftover nectar. However, the stigmas of Gesnerieae species remain receptive for nearly 30 straight hours, and pollen deposition was observed during hummingbird visits occurring at dawn and dusk. The corolla constriction appears to direct the hummingbird's bill to contact stamens and pistils in flowers that would otherwise be too wide for effective pollination to occur (video 1, Supporting Information). The constriction also makes nectar overflow accumulate as a nectar drop in the lower limb of the corolla, which is visible during the late night hours. This nectar drop may enhance the chances of pollination to unvisited flowers, by making nectar more accessible or attractive to bats. Future work should address the functional significance of corolla constriction in relation to all observed functional groups of pollinators.

Predictability of pollination syndromes: The current debate on pollination syndromes has focused on two major issues: the role of pollination specialization and the predictive power of syndromes (Fenster *et al.* 2004; Ollerton *et al.* 2007). The notion that syndromes reflect natural selection to enhance pollen transfer by principal pollinators assumes that specialization into functional groups of pollinators (*sensu* Armbruster *et al.* 2000) has been important in shaping floral evolution. We have demonstrated that floral characteristics in Antillean Gesneriaceae assemble species into hummingbird and bat pollination syndromes as well as into an intermediate floral phenotype that is closer to chiropterophily. Phylogenetic relatedness cannot account for all of the similarity among species that fell into particular syndrome categories (Zimmer *et al.* 2002, Martén-Rodríguez *et al*. unpublished phylogeny). For instance, the cluster that contains bat-pollinated specialists in the ordination includes at least three independent origins of this pollination system, while the cluster comprised by species with generalized pollination systems includes two independent origins. Hummingbird-pollinated species are distributed across at least four different clades, although in the tribe Gesnerieae hummingbird pollination is most likely ancestral (Martén-Rodríguez *et al*. unpublished phylogeny). Significant correlations among traits and pollinators (e.g. timing of anthesis, timing of nectar production, corolla shape and colour) suggest that sets of floral characters have responded to selection to enhance pollination by the observed visitor guilds. Support for syndromes has been found in various other plant taxa using multivariate approaches (Sakai *et al.* 1999, Wilson *et al.* 2004, Wolfe & Sowell 2006). For example, in Bornean gingers, three clusters of floral phenotypes defined in multivariate space corresponded with pollination by spiderhunters (Nectarinidae) and two different groups of bees (Anthophoridae and Halictidae; Sakai *et al.* 1999). Likewise, in *Penstemon* clustering of ornithophilous and melittophilous species strongly corresponded with the predicted pollinators (Wilson *et al.* 2004).

Predictability is the second major issue concerning the debate on pollination syndromes (Ollerton *et al*. 2007). Our statistical evaluation indicates that floral traits are good predictors of specialized hummingbird and bat pollination in Antillean Gesneriaceae. However, the classificatory scheme was not perfect: *Rhytidophyllum minus* (hummingbird-pollinated) and *Gesneria quisqueyana* (bat-pollinated) were both misclassified as generalists. The first species has been observed for only one year and although nocturnal observation time (18 hours) was within the range of other bat-pollinated species in our sample, it is possible that further observations will reveal the expected nocturnal pollinators. It is also possible that given the restricted present distribution of *R. minus* (one population in eastern Cuba isolated from undisturbed habitats), bat visits are rare and thus, the species relies largely on hummingbirds for reproduction. The second misclassified species, *G. quisqueyana,* prevents hummingbird visitation by an active exclusion mechanism: the two-night flowers of *G. quisqueyana* close up during daytime. Therefore bat specialization is achieved by a unique phenological trait not included in the statistical analysis. This finding is evidence that, even within groups of related species, the pathways to specialization vary, resulting in different phenotypes associated with the same pollination syndrome.

In contrast to specialized Gesnerieae species, suites of floral traits did not consistently predict generalized pollination systems (Table 3). However, the presence of a constricted bell-shaped corolla was, in most cases, a good indicator of generalization. The variability of other floral traits in the generalists may reflect the more variable selective regimes to which species with nocturnal and diurnal pollinators are exposed. Alternatively, certain associations may reflect phylogenetic affinities rather than pollinator-mediated selection. For example, in a multivariate analysis of South African asclepiads, Ollerton *et al.* (2003) found distinct separation of wasp and beetle pollination syndromes, but the generalist *Xysmalobium gerrardi* was grouped near its beetle-pollinated sister species.

In general, we found little specialization of floral visitors to particular floral phenotypes. From an ecological standpoint, the asymmetric specialization observed in the interaction between flowers and hummingbirds in the Gesnerieae supports recent findings of mutualistic networks of species. Asymmetric interactions commonly characterize plant-pollinator food webs and networks (Bascompte *et al.* 2005, 2006) and have also been described for clades of species with highly specialized pollination systems (e.g. the South African orchid clade Coryciinae, where a single species of oil-collecting bee pollinates 15 species of orchids; Pauw 2006). In the Antilles, most Gesnerieae species have relatively short flowering periods and restricted distributions, while the animal pollinators have broader geographic ranges and need to feed year round. These results indicate that the evolution of pollination specialization does not have to be reciprocal;

extreme specialization on the plant side has evolved without a corresponding specialization of the pollinators.

Because pollination syndromes are the result of convergent evolution across many different angiosperm flowers, they are not expected to describe the uniqueness of floral phenotypes evolved in different environments and phylogenetic backgrounds, nor can they be expected to predict unfailingly all floral visitors. This principle was stressed by the proponents of pollination syndromes (Faegri & van der Pjil 1978; Vogel 2006) and has been widely recognized by other researchers in the field (e.g. Stebbins 1970, Fenster *et al.* 2004, Ollerton *et al.* 2007). As exemplified in this study, pollination syndromes describe suites of traits that might evolve in a correlated way under selection by principal pollinators (Stebbins 1970). However, secondary floral visitors acting primarily as nectar and pollen robbers may also play important roles in floral evolution (Thompson 2003). Last, selection by different functional groups of pollinators may be responsible for the existence of floral phenotypes that appear intermediate or that do not fit classic syndromes. The study of ecological interactions between plants and different kinds of floral visitors, including mutualists and parasites, remains a major task to complete in order to elucidate the evolutionary processes responsible for the floral diversification of Antillean Gesnerieae and other groups of tropical plants.

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TABLE 1. Pollination system, visitation frequencies and pollinator importance values recorded for 23 species of Gesneriaceae from the Antillean islands (19 species) and Costa Rica (4 species). Pollinator visitation was calculated as the number of visits per flower per day. Mean values over the number of study years are presented followed by the range across years in brackets. Pollinator importance was calculated as the product of proportional visitation rates and a proxy for efficiency (proportion of contacts with anthers and stigmas). Importance values were scaled to proportions. Number of years and hours of observation are provided in the last column.

SPECIES	**Locality**	**Floral phenotype**	**Pollinator**	**Pollinator visitation**	**Scaled Importance**	**No of years**
				Mean [Range]	Mean [Range]	[No of hours]
Besleria solanoides	Costa Rica	Tubular	Hummingbird	3.0	0.97	**1** [19]
			Butterfly	0.1	0.03	
Capanea grandiflora	Costa Rica	Campanulate	Bat	1.7	1.00	**1** [43]
Columnea consanguinea	Costa Rica	Tubular	Hummingbird	4.2	1.00	**1** [13]
Columnea quercetii	Costa Rica	Tubular	Hummingbird	6.4	1.00	**1** [14]
Gesneria acaulis	Jamaica	Tubular	Hummingbird	0.3	1.00	**1** [6]
Gesneria calycosa	Jamaica	Campanulate	Bat	0.9	1.00	**1** [7]
Gesneria citrina	Puerto Rico	Tubular	Hummingbird	0.4 [0.3 – 0.5]	1.00	**3** [67]
Gesneria cuneifolia	Puerto Rico	Tubular	Hummingbird	0.5 [0.4 – 0.6]	1.00	**3** [51]
Gesneria fruticosa	Hispaniola	Campanulate	Bat	1.2 [0.9 – 1.6]	1.00	**2** [21]
Gesneria pedicellaris	Hispaniola	Tubular	Hummingbird	0.4 [0.3 – 0.5]	1.00	**2** [14]
Gesneria pedunculosa	Puerto Rico	Campanulate	Bat	3.9 [3.7 – 4.2]	0.84 [0.80 – 0.91]	**3** [90]
			Bananaquit	1.0 [0.7 – 1.6]	0.09 [0.08 – 0.09]	
			Diurnal insects	0.6 [0.0 – 0.9]	0.07 [0.00 – 0.14]	
Gesneria pulverulenta	Hispaniola	Tubular	Hummingbird	2.2 [1.7 - 2.7]	1.00	**2** [12]
Gesneria quisqueyana	Hispaniola	Subcampanulate	Bat	1.5 [0.9 – 2.1]	0.95 [0.90 – 1.00]	**2** [24]

			Hummingbird	0.05 [0.0 – 0.1]	0.05 [0.00 – 0.10]	
Gesneria reticulata	Puerto Rico	Tubular	Hummingbird	0.1 [0.0 – 0.2]	1.00	**3** [41]
Gesneria ventricosa	St. Lucia	Tubular	Hummingbird	0.5	1.00	**1** [7]
Gesneria viridiflora subsp. sintenisii	Puerto Rico	Subcampanulate	Bat	2.5 [1.3 – 3.6]	0.52 [0.32 – 0.72]	**2** [48]
			Hummingbird	3.0 [2.5 – 3.5]	0.42 [0.28 – 0.57]	
			Moth	3.3 [3.0 – 3.6]	0.06 [0.00 – 0.11]	
Pheidonocarpa corymbosa	Jamaica	Tubular	Hummingbird	2.0	1.00	**1** [6]
Rhytidophyllum asperum	Hispaniola	Tubular	Hummingbird	1.9 [1.5 – 2.1]	0.96 [0.93 – 1.00]	**3** [26]
			Diurnal insects	0.1 [0.0 – 0.2]	0.04 [0.00 – 0.07]	
Rhytidophyllum auriculatum	Puerto Rico	Subcampanulate yellow/ red	Bat	0.5 [0.0 – 1.2]	0.20 [0.00 – 0.44]	**2** [20]
			Bananaquit	0.2 [0.0 – 0.5]	0.10 [0.00 – 0.20]	
			Hummingbird	2.5 [1.7 – 3.4]	0.70 [0.56 – 0.80]	
Rhytidophyllum grandiflorum	Hispaniola	Subcampanulate yellow/ red	Hummingbird	3.0	0.27	**1** [13]
			Moth	13.7	0.23	
			Diurnal insects	9.7	0.50	
Rhytidophyllum leucomallon	Hispaniola	Subcampanulate yellow	Bat	2.4 [0.0 – 4.7]	0.24 [0.00 – 0.48]	**2** [25]
			Hummingbird	8.1 [6.0 – 11.9]	0.65 [0.44 – 0.86]	
			Moth	4.1 [1.3 – 6.9]	0.07 [0.03 – 0.12]	
			Diurnal insects	0.3 [0.3 – 0.4]	0.04 [0.03 – 0.04]	
Rhytidophyllum minus	Eastern Cuba	Subcampanulate yellow	Hummingbird	4.7	1.00	**1** [39]
Rhytidophyllum vernicosum	Hispaniola	Subcampanulate yellow/ red	Hummingbird	8.7 [7.0 – 10.4]	0.76 [0.75 – 0.77]	**2** [28]
			Moth	3.3 [1.5 – 5.0]	0.14 [0.09 – 0.19]	
			Diurnal insects	1.1 [0.5 – 1.7]	0.10 [0.04 – 0.16]	

TABLE 2. Spearman correlation coefficients among all floral traits of 23 Gesneriaceae species used for floral ordinations. Bolded numbers indicate significant correlations after sequential Bonferroni adjustment.

	PE	CWM	CC	CUR	NC	SYM	TAD	TNP	Colour	Spots
Corolla length (CL)	- 0.02	- 0.21	- 0.53	- 0.23	0.15	- 0.36	0.38	0.38	0.26	- 0.32
Pistil exsertion (PE)		0.30	- 0.07	0.26	- 0.16	**0.69**	- 0.25	- 0.25	- 0.37	- 0.01
Corolla width at mouth (CWM)			0.50	0.28	- 0.36	0.44	**- 0.84**	**- 0.85**	- 0.54	**0.70**
Corolla constriction (CC)				0.28	- 0.35	0.39	**- 0.65**	**- 0.65**	- 0.15	0.51
Corolla curvature (CUR)					- 0.27	0.56	- 0.11	- 0.11	0.08	0.42
Nectar concentration (NC)						- 0.24	0.48	0.48	0.00	- 0.55
Symmetry (SYM)							- 0.36	- 0.36	- 0.22	0.23
Timing anther dehiscence (TAD)								**1.00**	**0.66**	**- 0.73**
Timing nectar production (TNP)									**0.66**	**- 0.78**
Color										- 0.42

TABLE 3. Number of observations classified into expected pollination system and posterior probabilities under cross-validation of discriminant analysis of multivariate set of floral traits of 23 Gesneriaceae species.

Classified as A priori	Bat	Generalist	Hummingbird	Total	Posterior probability Error rate
Bat	4	1	0	5	0.210
	0.820	0.530			
Generalist	1	3	1	5	0.274
	0.670	0.714	0.731		
Hummingbird	0	1	12	13	0.021
		0.958	1.000		

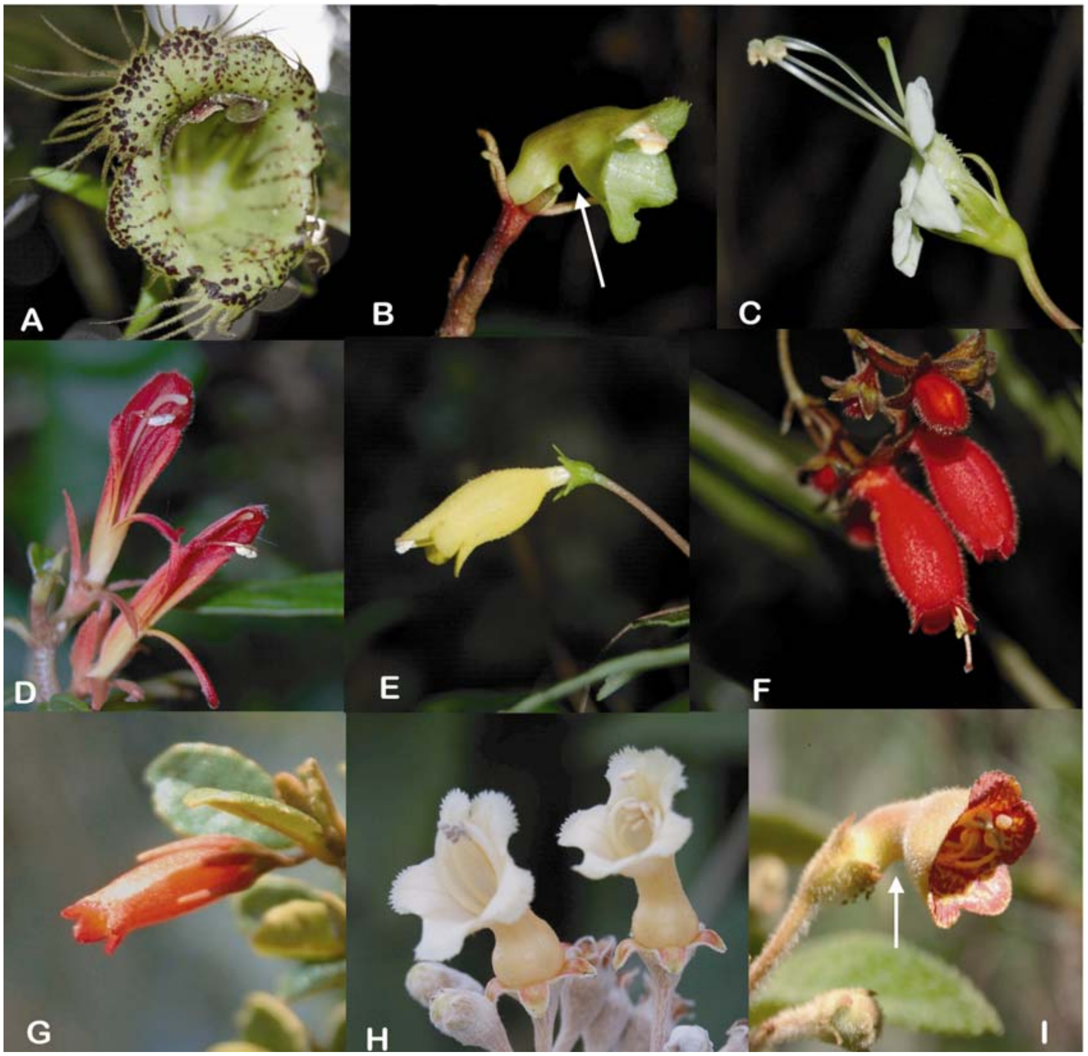
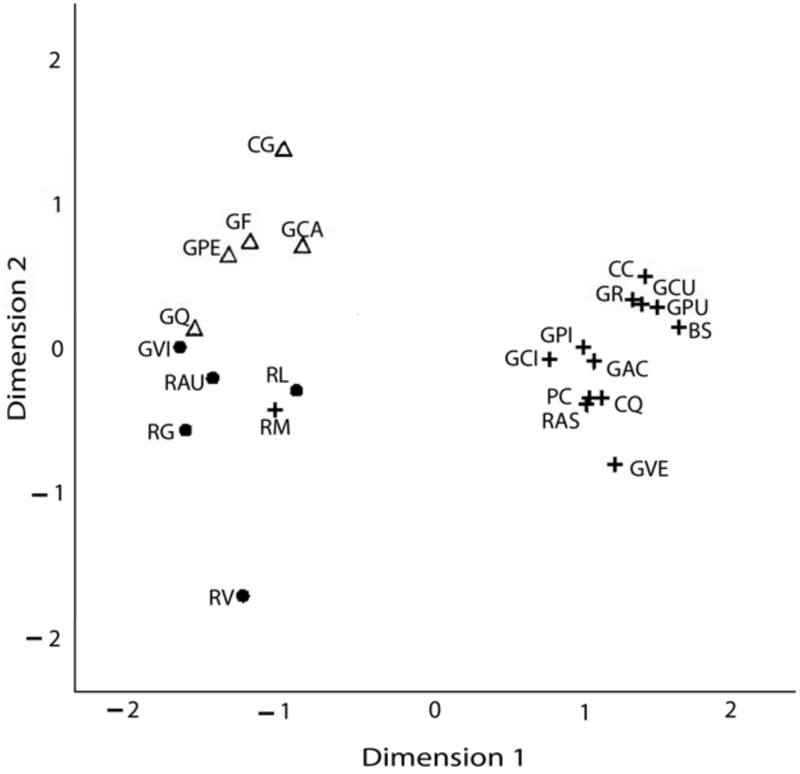
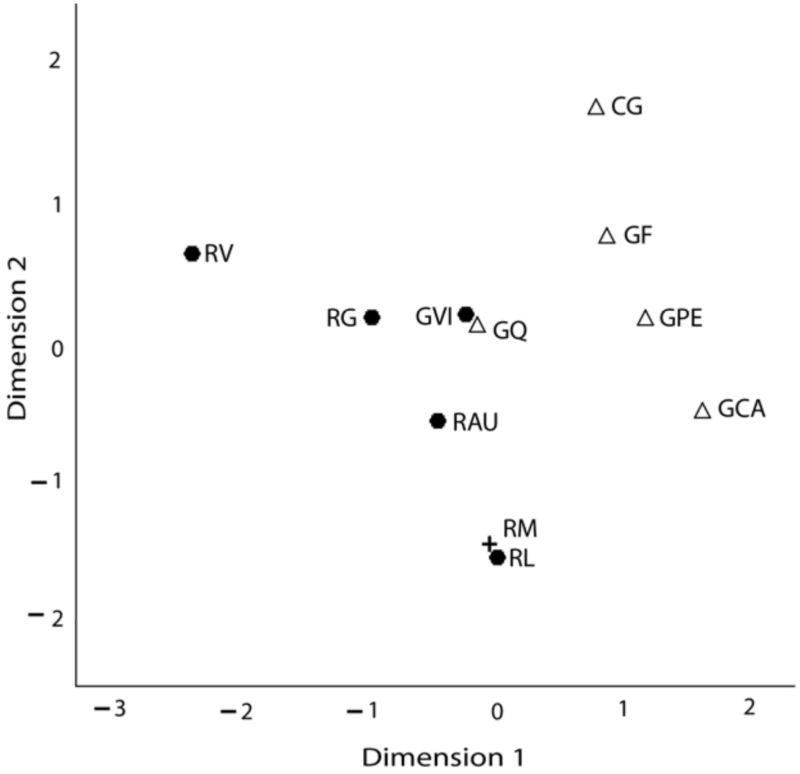


FIGURE 1. Three predominant floral phenotypes in Antillean Gesneriaceae. Photos A-C correspond to *Gesneria fruticosa, G. viridiflora* subsp. *sintenisii* and *G. pedunculosa*, representing the bat pollination syndrome (green or white bell-shaped flowers with nocturnal anthesis and high nocturnal nectar production). Photos D-G correspond to *Columnea quercetii, Gesneria citrina, G. decapleura* and *G. pulverulenta* representing the hummingbird pollination syndrome (tubular red or yellow corollas with diurnal anthesis and nectar production). Photos H-I correspond to *Rhytidophyllum leucomallon, R. vernicosum*, with mixed traits of diurnal and nocturnal pollination syndromes (yellow to spotted red bell-shaped flowers with nocturnal and/ or diurnal anthesis and nectar production). Corolla constriction indicated by white arrow.



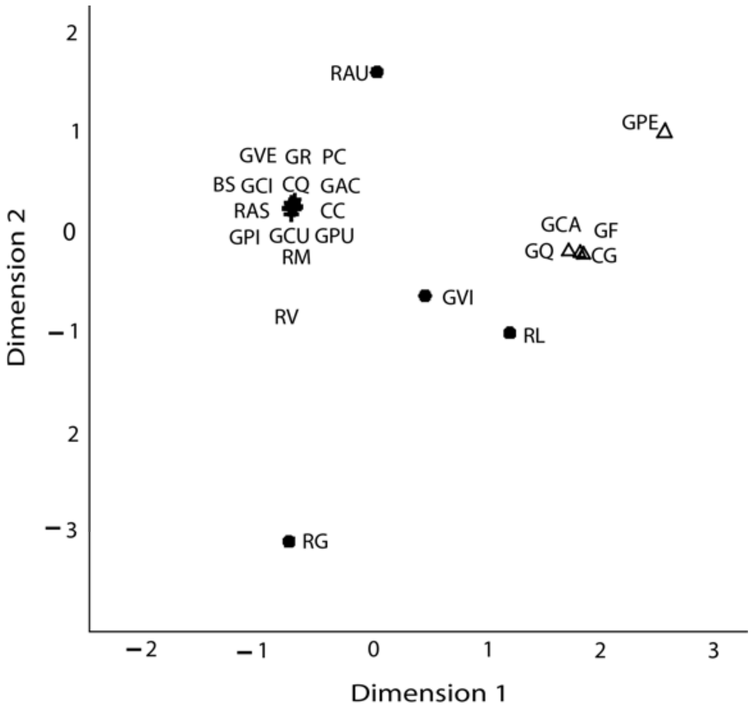
Floral trait	**Dim 1**	**Dim 2**
Corolla length ventral	0.449	0.252
Pistil exertion	- 0.328	- 0.079
Corolla width at mouth	**- 0.781**	0.012
Corolla constriction	**- 0.712**	- 0.456
Corolla curvature	- 0.478	- 0.565
Nectar concentration	**0.549**	- 0.115
Symmetry	**- 0.627**	- 0.537
Timing of anthesis	**0.851**	- 0.149
Timing of nectar	**0.851**	- 0.149
Colour	**0.503**	- 0.431
Spots	**- 0.812**	0.117
Pollinator Importance		
Hummingbird	**0.719**	- 0.297
Bananaquit	- 0.349	0.105
Bat	**- 0.575**	**0.555**
Moth	**- 0.500**	- 0.430
Diurnal insects	- 0.295	- 0.441

FIGURE 2. Multidimensional scaling analysis of 23 Gesneriaceae species based on 11 floral characters. Triangles represent species that specialize on bat pollination, plus signs represent species that were exclusively hummingbird-pollinated and dots represent species with mixed hummingbird and nocturnal pollination (bats and/or moths). Spearman correlation coefficients are listed for associations of dimensions 1 and 2, with floral traits and with pollinator importance values. Bolded coefficients indicate significant correlations following sequential Bonferroni adjustment ($p < 0.05$).



Floral trait	**Dim 1**	**Dim 2**
Corolla length ventral	0.345	0.228
Pistil exertion	0.596	- 0.182
Corolla width at mouth	0.068	0.687
Corolla constriction	**- 0.837**	- 0.418
Corolla curvature	- 0.670	0.373
Nectar concentration	0.176	- 0.200
Symmetry	0.200	- 0.500
Timing of anthesis	- 0.500	0.300
Timing of nectar	- 0.500	0.300
Colour	- 0.724	- 0.261
Spots	0.387	0.710
Pollinator importance		
Hummingbird	**- 0.719**	- 0.330
Bananaquit	0.027	- 0.242
Bat	**0.796**	0.278
Moth	- 0.642	0.084
Diurnal insects	- 0.357	0.094

FIGURE 3. Multidimensional scaling analysis of 11 Gesneriaceae species based on 11 floral characters (excluding 12 species with tubular flowers). *Rhytidophyllum minus* (RM, plus sign next to RL) was included because with only one year of observation, the occurrence of bat pollination cannot be discarded. As above, triangles represent bat-pollinated species and dots represent species with mixed hummingbird and nocturnal pollination (bats and/or moths). Spearman correlation coefficients are listed for associations of dimensions 1 and 2, with floral traits and with pollinator importance values. Bolded coefficients indicate significant correlations following sequential Bonferroni adjustment ($p < 0.05$).



Floral trait	**Dim 1**	**Dim 2**
Corolla length ventral	- 0.026	0.128
Pistil exertion	0.350	0.073
Corolla width at mouth	0.360	- 0.595
Corolla constriction	- 0.059	- 0.476
Corolla curvature	0.160	- 0.045
Nectar concentration	0.195	0.197
Symmetry	0.093	- 0.156
Timing of anthesis	- 0.549	0.504
Timing of nectar	- 0.608	0.514
Colour	**- 0.724**	0.192
Spots	0.381	- 0.331
Pollinator Importance		
Hummingbird	**- 0.555**	**0.573**
Bananaquit	0.357	**0.511**
Bat	**0.875**	- 0.333
Moth	- 0.177	**- 0.689**
Diurnal insects	- 0.397	- 0.414

FIGURE 4. Multidimensional scaling of 23 species of Gesneriaceae based on pollinator importance values. Importance was calculated as the product of visitation rates and effectiveness (contact with reproductive organs) and standardized as a proportional value. Note the hummingbird-pollinated species (plus sign) are mostly clustered in one point. Triangles indicate species primarily bat-pollinated and circles indicate generalist species. Spearman correlation coefficients are listed for associations of dimensions 1 and 2, with floral traits and with pollinator importance values. Bolded coefficients indicate significant correlations following sequential Bonferroni adjustment ($p < 0.05$).

Appendix I. Study locations for 23 species of Gesneriaceae studied in Costa Rica and five Antillean islands.

Species	Study site	Elevation m.s.l.	Geographic coordinates
Besleria solanoides Kunth	Costa Rica, Puntarenas, Monteverde, Estacion Biologica	1400	10°18' N, 84°48' W
Capanea grandiflora (Kunth) Decne. ex Planch.	Costa Rica, Alajuela, San Gerardo Biological Station	1200	10°21' 51" N, 84°47' 27" W
Columnea consanguinea Hanstein	Costa Rica, Alajuela, San Gerardo Biological Station	1200	10°21' 51" N, 84°47' 27" W
Columnea quercetii Oersted	Costa Rica, Alajuela, San Gerardo Biological Station	1200	10°21' 51" N, 84°47' 27" W
Gesneria acaulis Linnaeus	Jamaica, Windsor Research Center, Cockpit country	150	18°21' 05" N, 77° 38' 51" W
Gesneria calycosa (W. Hooker) O. Kuntze	Jamaica, Windsor Research Center, Cockpit country	150	18°21' 05" N, 77° 38' 51" W
Gesneria citrina Urban	Puerto Rico, Arecibo Observatory and Rio Abajo	300	18°20' 36" N, 66°45' 11" W
Gesneria cuneifolia (A.P. de Candolle) Fritsch	Puerto Rico, Arecibo Observatory, Rio Abajo	300	18°20' 36" N, 66°45' 11" W
Gesneria fruticosa (Linnaeus) O. Kuntze	Dominican Republic, Cordillera Central, EL Manaclar	1100	19°35' N, 70° 45' W.
Gesneria pedicellaris Alain	Dominican Republic, Cordillera Septentrional, Blanco Arriba, La Jibara	680	19°30' N, 70° 20' W.
Gesneria pedunculosa (A.P. de Candolle) Fritsch	Puerto Rico, Arecibo Observatory	300	18°20' 36" N, 66°45' 11" W
Gesneria pulverulenta Alain	Dominican Republic, Parque Nacional Sierra de Bahoruco, Hoyo de Pelempito	1200-1250	18°05' N, 71°30' W
Gesneria quisqueyana Alain	Dominican Republic, Cordillera Septentrional, Blanco Arriba, La Jibara	680	19°30' N, 70°20' W.
Gesneria reticulata (Grisebach) Urban	Puerto Rico, El Yunke National Forest	600	18°18' N, 65°46' W
Gesneria ventricosa Swartz	St. Lucia: Edmund Forest	500	
Gesneria viridiflora subsp. *sintenisii* (Urban) L. Skog	Puerto Rico, El Yunke National Forest	700 - 800	18°18' N, 65°47' W
Pheidonocarpa corymbosa (Swartz) L.E. Skog	Jamaica: Blue Mountains, Cane River Falls	200	17°57' 54" N, 76°41' 36" W
Rhytidophyllum asperum Alain	Dominican Republic, Parque Nacional Sierra de Bahoruco, Hoyo de Pelempito	1200	18°05' N, 71°30' W
Rhytidophyllum auriculatum W. Hooker	Puerto Rico, Cordillera Central, Toro Negro	1150	18°09' 05" N, 66°33' 14" W
Rhytidophyllum grandiflorum ined.	Dominican Republic, Sierra de Neiba, Carretera Internacional	1700	18°41' N, 70°46' W
Rhytidophyllum leucomallon Hanstein	Dominican Republic, Parque Nacional Sierra de Bahoruco, Aceitillar	650	18°06' N, 71°37' W
Rhytidophyllum minus Urban	Cuba, Santiago, Castillo El Morro	50	18°58' 06" N, 75°52' 10" W
Rhytidophyllum vernicosum Urban & Ekman	Dominican Republic, Parque Nacional Sierra de Bahoruco, road to Caseta 2	1900	18°12' 32" N, 71°33' 28" W

Appendix II. Mean values for 11 morphological and phenological floral traits of 23 species of Gesneriaceae from Costa Rica and five Antillean islands. Trait code values are as follows: CL for corolla length, PE for pistil exertion, CWM for corolla width at the mouth, CC for corolla constriction, CUR for corolla curvature, NC for nectar concentration, SYM for symmetry: (0) subactinomorphic, (1) zygomorphic, TA for timing of anthesis and TNP for timing of nectar production: (0) mostly nocturnal, (1) 24 hours, (2) mostly diurnal. Color codes are: (1) green, (2) yellow, (3) orange, (4) red. Spot codes denote: (0) absence, (1) presence of dark red markings on inner corolla.

SPECIES	**CL**	**PE**	**CWM**	**CC**	**CUR**	**NC**	**SYM**	**TA**	**TNP**	**Color**	**Spots**
Besleria solanoides	14.0	-4.1	2.8	0	0.0	24	0	2	2	3	0
Columnea consanguinea	21.1	-4.2	3.6	0	1.7	25.3	0	2	2	2	0
Capanea grandiflora	39.7	-3.7	16.0	0	8.8	15.8	0	0	0	1	1
Columnea quercetii	31.4	-2.3	4.8	0	10.3	25.0	1	2	2	3	0
Gesneria acaulis	27.4	0.1	3.6	0	17.7	14.5	1	2	2	4	0
Gesneria calycosa	20.9	18.9	12.0	0	5.0	11.2	1	0	0	1	0
Gesneria citrina	21.0	2.8	3.4	0	12.1	13.0	1	2	2	2	0
Gesneria cuneifolia	26.2	-4.2	2.7	0	9.9	12.8	0	2	2	4	0
Gesneria fruticosa	29.7	1.7	15.0	0	3.3	8.9	1	0	0	1	1
Gesneria pedunculosa	18.1	19.0	8.3	0	20.9	10.6	1	0	0	1	1
Gesneria pedicellaris	27.1	0.8	3.9	0	3.7	14.5	1	2	2	4	0
Gesneria pulverulenta	15.7	-1.5	3.5	0	2.4	.	0	2	2	4	0
Gesneria quisqueyana	21.0	-3.7	10.0	1	13.1	11.4	1	0	0	1	1
Gesneria reticulata	19.1	-5.3	2.5	0	3.3	8.3	0	2	2	4	0
Gesneria ventricosa	34.8	18.0	5.6	0	41.5	14.8	1	2	2	3	0
Gesneria viridiflora	18.1	0.7	10.0	1	31.3	12.1	1	0	0	1	1
Pheidonocarpa corymbosa	17.2	-1.1	3.3	0	17.7	18.2	1	2	2	3	0
Rhytidophyllum asperum	24.2	-0.3	4.4	0	24.7	14.8	1	2	2	3	0
Rhytidophyllum auriculatum	19.7	-0.7	8.5	1	15.2	10.8	1	0	0	2	1
Rhytidophyllum grandiflorum	26.1	-3.0	12.6	1	35.9	9.0	1	0	0	4	1
Rhytidophyllum leucomallon	20.6	-0.1	7.8	1	1.2	15.2	1	0	0	2	0
Rhytidophyllum minus	19.5	0.1	10.7	1	5.0	.	1	0	0	2	0
Rhytidophyllum vernicosum	28.5	-2.7	11.5	1	47.0	9.2	1	1	1	4	1

Appendix III. List of native pollinators (visitors observed contacting the flower's reproductive organs), non-pollinating visitors (animals that did not contact the flower's reproductive organs) and introduced pollinators (††) observed on 23 species of Gesneriaceae from the Antilles and Costa Rica (CR). Hummingbirds (family Trochilidae) were identified using field guides. For nectar-feeding bats (family Phyllostomidae) the most likely species were inferred from size (observed in the video clips) and geographic distribution. By these criteria the brown flower bat, *Erophylla sezekorni,* is also a possible visitor to the small *Gesneria* and *Rhytidophyllum* flowers visited by *Monophyllus redmanii,* however due to its larger size, it is perhaps less common. Four of the Gesnerieae species listed below have pollinators documented only from anecdotal observations; these species (†) were not included in the multivariate analysis.

SPECIES	**Pollinator**	**Non-pollinating and introduced visitors ††**
Besleria solanoides (CR)	*Lampornis calolaema* (Trochilidae) Butterfly (1 species)	
Capanea grandiflora (CR)	Bat –unknown species	
Columnea consanguinea (CR)	*Heliodoxa jacula* (Trochilidae)	
Columnea quercetii (CR)	*Lampornis hemileuca* (Trochilidae)	
Gesneria acaulis	*Anthracothorax mango* (Trochilidae)	
Gesneria calycosa	*Phyllonycteris aphylla* (Phyllostomidae)	
Gesneria citrina	*Anthracothorax viridis, Chlorostilbon maugaeus* (Trochilidae)	*Coereba flaveola* (Coerebidae)
Gesneria cubensis †	*Anthracothorax dominicus* (Trochilidae)	
Gesneria cuneifolia	*Chlorostilbon maugaeus* (Trochilidae)	*Coereba flaveola* (Coerebidae)
Gesneria ekmanii †	Moths (various morphospecies)	
Gesneria fruticosa	*Phyllonicteris poeyi* (Phyllostomidae)	
Gesneria jamaicensis †	*Anthracothorax dominicus,* *Chlorostilbon swainsonii* (Trochilidae)	

SPECIES	**Pollinator**	**Non-pollinating and introduced visitors** ††
Gesneria pedicellaris	*Anthracothorax dominicus,* *Chlorostilbon swainsonii* (Trochilidae)	
Gesneria pedunculosa	*Monophyllus redmanii** (Phyllostomidae) *Coereba flaveola* (Coerebidae) Syrphid flies (2 morphospecies)	*Chlorostilbon maugaeus* Sphingid moth (1 morphospecies) *Apis mellifera*††
Gesneria pulverulenta	*Chlorostilbon swainsonii* (Trochilidae)	
Gesneria quisqueyana	*Monophyllus redmanii** (Phyllostomidae) *Chlorostilbon swainsonii* (Trochilidae)	Ants
Gesneria reticulata	*Chlorostilbon maugaeus* (Trochilidae)	
Gesneria ventricosa	*Eulampis sp.* (Trochilidae)	*Orthorhyncus cristatus* (Trochilidae)
Gesneria viridiflora subsp. sintenisii	*Chlorostilbon maugaeus* (Trochilidae) *Monophyllus redmanii** (Phyllostomidae) Moths (various morphospecies)	Ants Grasshoppers
Pheidonocarpa corymbosa	*Trochilus* sp. (Trochilidae)	
Rhytidophyllum asperum	*Chlorostilbon swainsonii* (Trochilidae) Halictid bees (one morphospecies)	
Rhytidophyllum auriculatum	*Chlorostilbon maugaeus* (Trochilidae) *Monophyllus redmanii** (Phyllostomidae) *Coereba flaveola* (Coerebidae)	*Apis mellifera*††
Rhytidophyllum berteroanum †	*Chlorostilbon swainsonii* (Trochilidae)	
Rhytidophyllum grandiflorum	*Chlorostilbon swainsonii* (Trochilidae) Moths (various morphospecies) Syrphid flies (2 morphospecies) Small bees (1 species)	*Apis mellifera*††
Rhytidophyllum leucomallon	*Chlorostilbon swainsonii* (Trochilidae) *Monophyllus redmanii** (Phyllostomidae) Moths (various morphospecies) Syrphid and Tachinid flies (2 morphospecies); Halictid bees (1 sp.)	*Apis mellifera*††

SPECIES	**Pollinator**	**Non-pollinating and introduced visitors** [††]
Rhytidophyllum minus	*Chlorostilbon ricordii*	*Apis mellifera*[††]
Rhytidophyllum vernicosum	*Chlorostilbon swainsonii* (Trochilidae) Moths (various morphospecies) Syrphid flies (3 morphospecies), Tachinid flies (1species) Small bees (2 morphospecies)	Staphylinid beetles (1 species)

CHAPTER 3

Pollen Limitation and Reproductive Assurance in Antillean Gesnerieae: are specialists more vulnerable to reproductive failure than their generalist congeners?

Co-authored with Charles B. Fenster

Abstract

Pollen limitation of plant female fecundity occurs when pollen transfer is insufficient to fertilize all ovules that could develop into seed with the available resources. Pollen limitation has been widely documented in angiosperms and recently suggested to be associated with floral specialization. This study uses a group of Antillean Gesneriaceae with contrasting pollination systems (bat, hummingbird and generalist) to assess the premise that plants with specialized pollination systems and infrequent floral visitation experience greater pollen limitation of fruit and seed set than their generalist congeners. Alternatively, specialists may possess mechanisms that reduce pollen limitation, such as autonomous self-pollination. Tests for pollen limitation were conducted on nine Gesnerieae species from the Dominican Republic and Puerto Rico during 2006 and 2007. A comparison of pollen supplementation and open pollination treatments provided evidence for significant pollen limitation associated with specialized Gesnerieae, including both bat and hummingbird-pollinated species; no pollen limitation was detected in any of the four generalist Gesnerieae species. No pollen limitation was detected in two ornithophilous *Gesneria* species with low hummingbird visitation but high potential and actual levels of autonomous self-pollination. A comparison of fruit and seed set between emasculated and unmanipulated flowers provided evidence for autonomous selfing acting as a reproductive assurance mechanism in ornithophilous *G.citrina, G. cuneifolia* and *G. reticulata*. The Puerto Rican population of *G. reticulata* relies almost exclusively on self-pollination for reproduction. A survey of autonomous self-pollination conducted on 15 Gesnerieae species revealed no significant association between reproductive assurance mechanisms and pollination system specialization. However, high potential autonomous selfing rates were only found among hummingbird-pollinated, tubular-flowered Gesnerieae, suggesting that flower morphology in addition to low pollinator visitation may facilitate the evolution of reproductive assurance mechanisms. This study provides support for the idea that generalized pollination systems might, in some cases, buffer against fluctuations in the pollinator environment. Furthermore, the findings of this study suggest that reproductive assurance mechanisms may allow the maintenance of highly specialized pollination systems in pollinator depauperate environments.

Keywords: Autonomous self-pollination, Gesneriaceae, Dominican Republic, pollen limitation, Puerto Rico, reproductive assurance.

Introduction

Most flowering plants rely on external agents to produce outcrossed seed. Consequently, failure in the process of pollen transfer among conspecifics can severely impact plant female reproductive success (Wilcock and Neiland 2002). Evidence for widespread pollen limitation has been found in recent surveys reflecting inadequacies in pollen supply that prevent full seed set, even when resources are plentiful (Burd 1994, Larson and Barrett 2000, Knight et al. 2005). The prevalence of pollen limitation across unrelated angiosperm lineages suggests that pollinators frequently do not effect successful cross-fertilization (Ashman et al. 2004, Aizen and Harder 2007). A potentially important determinant of pollination success is the degree of floral specialization, i.e., how dependent a plant species is on particular species or guilds of pollinators. However, the association between floral specialization and pollen limitation has only been assessed in a few literature and community-level surveys (Larson and Barrett 2000, Knight et al. 2005; Merrett et al. 2007) and not through direct comparisons within groups of related species.

Floral specialization has traditionally been considered an evolutionary pathway to promote effective outcross pollen transfer (Stebbins 1970, Faegri and van der Pjil 1978, Fenster et al. 2004). However, specialization can make plants vulnerable to temporal fluctuations in pollinator visitation or permanent loss of important pollinators (Wilcock and Neiland 2002). In contrast, plants with generalized flowers are buffered against fluctuations of individual pollinator species and are thus assumed to experience less variation of reproductive success than specialists (Waser et al. 1996). These views underlie the prediction that female fertility in specialized species should be more pollen limited than in generalists (Larson and Barrett 2000), and would suggest that the evolution of pollination specialization reflects selection acting to increase male rather than female reproductive success. Support for the prediction of increased pollen limitation in species with specialized pollination systems was found in two recent surveys, a meta-analysis of pollen limitation in flowering plants (Knight et al. 2005) and a field study of a shrub assemblage in New Zealand (Merrett et al. 2007). Deviations from this trend are expected to derive from variation in pollinator efficiency, since pollen limitation may occur due to insufficient pollen transfer and deposition of low quality pollen, i.e., genetically related, incompatible, or heterospecific pollen (Aizen and Harder 2007). Thus, generalized species could also experience pollen limitation if floral visitors do not transfer adequate amounts of compatible pollen (Wilcock and Neiland 2002). Comparisons of pollen limitation among closely related species in similar ecological settings but with varying degrees of floral specialization are essential to evaluate these contrasting hypotheses.

The examination of pollen limitation patterns within groups of related species allows us to examine the frequently overlooked association between pollination and breeding system evolution. For instance, when plants experience intense pollen limitation of female fertility, natural selection should favor traits or trait combinations that increase attraction of existing pollinators (e.g. Hansen and Totland 2006), attract more pollinator species, and / or decrease pollinator dependence (e.g. Fishman and Willis 2008). Thus, autonomous breeding systems may evolve to reduce reliance on unpredictable or ineffective pollinators (Darwin 1877, Lloyd 1979). Available evidence shows that autonomous selfing mechanisms are widespread across different angiosperm lineages encompassing a wide variety of pollination systems (Lloyd and Schoen 1992, Fenster and Martén-Rodríguez 2007). However, the pattern of association between levels of floral specialization and the evolution of autonomous self-pollination remains unclear (Fenster and Martén-Rodríguez 2007). This study evaluates the hypothesis that specialization increases vulnerability to pollination failure and makes plants more likely to exhibit reproductive assurance mechanisms in a group of closely related Gesneriaceae.

The study species belong in the tribe Gesnerieae, a clade of ~ 75 species that radiated in the Antilles from a single common ancestor (Zimmer et al. 2002). Based on field observations of pollinators (Martén-Rodríguez et al. in press), we estimate that approximately 60% of Gesnerieae species have highly specialized hummingbird pollination systems. The remaining species are primarily bat specialists or have generalized pollination systems (bats, birds and insects), and a few species are pollinated by bees. Pollinator visitation to specialized species is on average 10 times lower than visitation to generalized species (see study species descriptions), which provides the basis for our prediction that specialists should experience greater levels of pollen limitation or have reproductive assurance mechanisms. We used a group of Gesnerieae species with contrasting pollination systems (hummingbird, bat and generalist) to address the following questions: (1) Is female fertility in Gesnerieae limited by pollen availability? If so, (2) Is the occurrence and magnitude of pollen limitation associated with pollination specialization? (3) Are autonomous selfing mechanisms more common in species with specialized pollination systems? (4) Do autonomous selfing mechanisms provide reproductive assurance in hummingbird-pollinated Gesnerieae species? We predict that species with specialized pollination systems and infrequent pollinator service will exhibit autonomous selfing mechanisms providing greater reproductive assurance than species with frequent pollinator visitation. We also predict that specialized Gesnerieae species will experience greater pollen limitation of fruit and seed set than their generalist relatives.

Methods

Study sites and species descriptions – All members of the tribe Gesnerieae are perennial plants that range from rosettes to small trees. All species have woody stems, at least at the base of the plant (Skog 1976). The flowers are gamopetalous (fused petals) and they fall primarily into three main floral phenotypes: tubular, campanulate (bell-shaped), and subcampanulate (narrow bell-shaped with a corolla constriction above the nectar chamber). Pollinator observations of a subset of Gesnerieae species across the Antilles revealed that tubular-flowered species are pollinated exclusively by one to two species of hummingbirds, while campanulate (bell-shaped) flowered species are pollinated primarily by the bat *Monophyllus redmanii* (Martén-Rodríguez and Fenster 2008, and Martén-Rodríguez et al. in press). We designate levels of specialization following Fenster et al. (2004), where pollinators fall into functional groups, generally defined by taxonomic relatedness and the potential to exert similar selective pressures on flower structure (e.g., hummingbirds or nectar-feeding bats). Consequently, we consider Gesnerieae species pollinated primarily by hummingbirds or bat as specialists. We define generalization as effective pollination by two or more functional groups. Generalized Gesnerieae include species with subcampanulate flowers (bell-shaped with a corolla constriction), since they receive visits from different animal guilds – including bats, birds and insects – all of which have the potential to transfer pollen. The frequency of pollinator visits is significantly lower in species with specialized pollination systems than in generalized species (Kruskal-Wallis Test x^2=11.1, P =0.004, df =2; Hummingbird: visits/ flower/day Mean =1 ± 1.5 SEM, n= 9; Bat: Mean = 2 ± 1.8, n=5; Generalist: Mean

=13 ± 1.8, n=5, where n is the number of species). These estimates, calculated from Martén-Rodríguez et al. (in press), provide the basis for our prediction that pollen limitation should be higher in specialized Gesnerieae. Study sites and plant characteristics are listed in Appendix I, pollination systems are listed in Appendix II.

Our preliminary molecular and morphological phylogenies suggest that bat and generalized pollination systems are derived from hummingbird pollination, with at least two origins of bat and two origins of generalized flowers (Martén-Rodríguez et al. unpublished manuscript). Gesnerieae species are self-compatible and do not produce seed by apomixis (Martén-Rodríguez and Fenster 2008, and unpublished data). Breeding systems vary in the temporal separation and spatial placement of the reproductive organs. Most study species are protogynous (except for *Gesneria reticulata* - adichogamous, and *G. pedunculosa* - protandrous). The female phase in protogynous Gesnerieae lasts two to three days and anther dehiscence generally occurs by the second day. The spatial separation of anthers and stigmas observed the first day usually decreases by the time of anther dehiscence; therefore both temporal and spatial overlap between male and female functions provide the potential for delayed selfing. However, in most species with bell-shaped flowers, contact between the stigmatic surface and pollen is prevented by the specific positioning of the reproductive organs, with anthers contacting only the back side of the stigma. This type of placement of anthers and stigma is less common in tubular flowers.

Pollen limitation field experiments- To determine whether pollination system is associated with the degree of pollen limitation, we selected nine Gesnerieae species that encompass the three main pollination modes described above. Five species were studied in Puerto Rico (January –March, 2006 and 2007) and four in the Dominican Republic (hereafter DR; May- August, 2006 and 2007). Given that sister species pairs were not available at particular sites or in large enough numbers for experimentation, we ensured some level of phylogenetic independence by selecting, at each of three sites, at least a pair of related species with contrasting pollination systems (according to our unpublished phylogeny). The groups include: (1) *Rhytidophyllum vernicosum* (generalist moth and hummingbird pollination), *R. leucomallon* (generalist bat, hummingbird and moth pollination) and *Rhytidophyllum asperum* (hummingbird specialist); these species are shrubs that occur in Sierra de Bahoruco National Park, DR (18°06' N, 71°37' W -18°12' N, 71°33' W); (2) *G. pedunculosa* (bat pollination), *G. citrina* (hummingbird pollination) and *G. cuneifolia* (hummingbird pollination); these three species occur in the karst hills surrounding the Arecibo Observatory, Puerto Rico (18°20' N, 66°45' W), (3) *G. viridiflora* (generalist bat, hummingbird, moth pollination) and *G. reticulata* (self / hummingbird pollination) from the rainforest of El Yunke National Forest, Puerto Rico (18°18' N, 65°46' W). We also included *G. quisqueyana* (bat pollination) from Cordillera Septentrional in the DR (19°30' N, 70°20' W), because it is sister to *G. viridiflora* (our unpublished phylogeny), and it allows a direct specialist vs. generalist sister taxa comparison. The two species are morphologically similar and they occur at similar elevations with comparable weather conditions; however, the flowers of *G. quisqueyana* open only during the night (making this species a strictly bat specialist), whereas *G. viridiflora* flowers are open both day and night (Martén-Rodríguez et al. in press).

We tagged 21 to 33 pairs of plants per species; pairs of plants included a diverse representation of the different sizes and presumably ages of plants found in each population. Each pair consisted of individuals of similar size growing at least five m from each other. Within a pair, plants were assigned at random to a pollen supplementation treatment or an open pollination control. All flowers produced by an individual were subject to the same treatment. Pollen-supplemented flowers received a mixture of pollen from at least two different donors. Donor plants were located at least seven m away from the recipient plant to prevent mating between close relatives. Hand pollinations were conducted by rubbing dehisced anthers onto receptive stigmas. Timing of stigma receptivity and anther dehiscence were previously determined using the peroxidase test (Kearns and Inouye 1993). Open-pollinated flowers were unmanipulated to serve as controls for natural pollination. Because the study species are perennials and flowering generally lasts several weeks or months, field work was conducted for 14-27 days, depending on the per-day flower output of the species, i.e., species with longer flowering seasons and lower daily flower production (1-2 flowers) were studied for longer periods to ensure adequate sample sizes. Ideally, measurements should be taken over the entire lifespan of the plant (Ashman et al. 2004), but there are obvious logistical difficulties for implementing this approach on long-lived perennials. Thus, to reduce variation in potential conflicts of resource allocation with fruits developed before and after the study period, we removed mature fruits at the time of tagging and large flower buds present at the end of the experiment. By taking a sample representative of the population size and age classes, applying treatments to entire plants, and measuring fruit and seed set on the same individuals for two years, we expect to provide representative estimates of pollen limitation for each population during the study period, as well as a uniform setting to compare species with different degrees of pollination specialization.

Fruits were counted upon maturity (six to nine weeks later) and seed mass was measured in the lab. Seed mass was used as a surrogate for seed set due to the large number of seeds contained in each capsule. We previously determined seed mass to be highly correlated with seed number in the study species (Martén-Rodríguez and Fenster 2008). A measure of whole-plant seed production was calculated by multiplying mean fruit set by mean seed mass per plant; we calculated the pollen limitation index as [1-(open / pollen-supplemented whole-plant seed mass)] after Larson and Barrett (2000).

We tested for pollen limitation of fruit set and seed mass with repeated measures ANOVA, using proc GLIMMIX in SAS (SAS Institute 2004). Model terms included treatment (*open* vs. *pollen-supplemented*), specified as a fixed effect; year, specified as a random effect; and plant, as the subject of repeated measures. Degrees of freedom were estimated using the Satterthwaite option. This model was selected because particular treatments were applied to the same plants both years. The repeated measures approach accounts for the potential covariation resulting from taking two measurements on the same individuals. Furthermore, this approach provides a single random estimate of pollen limitation for each plant, which represents a sample taken across a wide range of age classes within the population. Differences in levels of pollen limitation between specialized and generalized species were evaluated with a Wilcoxon rank test (proc NPAR1WAY); the EXACT option was specified to obtain exact p-values because the normal approximation may not be accurate with small sample sizes. We excluded the two species with high levels of autonomous self-pollination to avoid a comparison of pollen limitation that was confounded by variation in the breeding system (i.e., the ability to self-pollinate).

Autonomous self-pollination survey - We compiled data for 15 Gesnerieae species to determine whether the degree of autonomous self-pollination is associated with particular pollination systems. The data set includes nine species from the DR and one from Puerto Rico surveyed in 2006- 2007, and five species of Puerto Rican *Gesneria* reported in an earlier study (Martén-Rodríguez and Fenster 2008). To determine the potential for autonomous selfing two to 10 flowers per plant were bagged in six to 30 individuals per species, depending on the population density. A comparable set of hand-pollinated outcrossed flowers was included to account for the variability in fruit production due to environmental or physiological conditions. Bridal veil fabric bags were used to cover flowers until fruit development. Mature fruits were counted six to nine weeks later. We report two measures of potential autonomous self-pollination: the fruit set of bagged flowers, and the autofertility index proposed by Lloyd and Schoen (1992), hereafter designated as AFI. This index is calculated by dividing the fruit set of bagged flowers by the fruit set of hand-outcrossed flowers.

To test for differences in the levels of autonomous self-pollination between specialized (hummingbird, bat) and generalized pollination systems we conducted non-parametric Wilcoxon rank tests (proc NPAR1WAY). We used the same test to evaluate differences in levels of autonomous selfing between species with tubular and species with bell-shaped flowers (campanulate and subcampanulate); as above the EXACT option was used to obtain p-values.

Reproductive assurance field experiments- In our sample, only hummingbird-pollinated Gesnerieae had the potential to set seed in the absence of pollinators (see below). To determine whether hummingbird-pollinated Gesnerieae actually use autonomous selfing as a reproductive assurance mechanism, we selected four species with autofertility indices greater than 10 % (fruit set from bagged flowers / hand-pollinated flowers; Table 3), and large population sizes to allow for adequate sampling. The species were tubular-flowered *G. citrina*, *G. cuneifolia*, *G. reticulata* and *R. asperum.* Prior to the start of these experiments, we tested for the effects of emasculation on two flowers of 10-15 plants per species by comparing two treatments: (1) hand-pollination, no emasculation (2) emasculation and hand-pollination. No significant differences in number of mature fruit or flower longevity were found between treatments, indicating emasculation has no negative effects on fruit production for these species.

Reproductive assurance experiments were conducted in 2006 and 2007 on 21 to 33 individuals per species. We followed plants for two to three weeks, tagging all flower buds and assigning them as pairs at random to the following treatments: (1) emasculation: flowers had their anthers removed in bud and were left uncovered to allow for pollinator visitation (a measure of geitonogamy and outcrossing), (2) control: flower buds were tagged and left intact to serve as a measure of natural pollination (geitonogamy, outcrossing and autonomous self-pollination). The contrast between the two treatments (control – emasculation) reflects the contribution of self-pollination. Fruit set was determined six to nine weeks after flowering and seeds were extracted and weighed on to the nearest 0.001 gram. Tests of the reproductive assurance hypothesis were conducted by comparing control and emasculated treatments for each hummingbird-pollinated species. We used the GLIMMIX procedure with treatment, year and their interaction specified as fixed effects in the model, and fruit set (fruits/ flowers) and seed mass set as the response variables. The covariance between emasculated and control treatments applied to the same individuals was accounted for by a random residual statement where plant was stated as the subject of repeated measures. Differences between emasculated and open pollination treatments within each year were tested using orthogonal contrast statements.

Results

Pollen limitation- We detected pollen limitation in four species with specialized pollination systems and no autonomous selfing, while we found no evidence for pollen limitation in five species that had generalized pollination systems or autonomous selfing mechanisms (Figure 1, Table 1). Specifically, we documented significant differences in fruit set and seed mass between pollen-supplemented and open pollination treatments for bat specialist *G. pedunculosa* and hummingbird specialists *G. citrina* and *R. asperum.* Pollen limitation of fruit set, but not seed set was detected for the bat specialist, *G. quisqueyana.* There were no significant differences between pollen supplementation and open pollination treatments for any of the three species with mixed pollination systems, i.e., *G. viridiflora, R. vernicosum* and *R. leucomallon*. No pollen limitation was detected for *G. cuneifolia* and *G. reticulata,* the two species with ornithophilous flowers but high levels of autonomous self-pollination. Pollen limitation was not associated with location as both pollen-limited and non-pollen limited species were found at all sites (Figure 1).

The degree of pollen limitation, as determined by the pollen limitation index (PL), was highest (above 25% difference between pollen-supplemented and open pollination) in bat-pollinated *Gesneria* and the two species with hummingbird-pollinated flowers and low reproductive assurance (Table 2). Intermediate values of pollen limitation (8-26 %) were characteristic of most other species, while no pollen limitation was detected for autogamous *G. reticulata*. However, outcrossed seed set was reduced by 97% in *G. reticulata* when the opportunity for self-pollination was eliminated (Table 2). Despite the small sample size (four specialists and three generalists with low or no autonomous selfing) we detected a trend for higher pollen limitation in the specialized species (Wilcoxon two-sample test, p= 0.0571).

Autonomous Self-pollination Survey- The ability to set seed autonomously was predominantly associated with hummingbird pollination, but there was great variation among species (Table 3). Autonomous fruit set upon bagging for hummingbird specialists ranged between 0% and 90%, and the autofertility index (AFI) was equally dispersed (0 - 0.98). In contrast, autonomous fruit set for bat-pollinated and generalist species ranged between 0% and 8%, with AFI values no greater than 0.09, indicating low or no potential for autonomous self-pollination. There were no differences in the autofertility index between specialized and generalized species (Wilcoxon two-sample test, p= 0.51). However, species with tubular flowers had significantly higher autofertility levels than species with bell-shaped flowers (Wilcoxon two-sample test, p= 0.017). These results partly support our prediction that specialization is associated with autonomous breeding systems.

Reproductive Assurance hypothesis of hummingbird-pollinated species- Autonomous self-pollination significantly increased fruit set in three ornithophilous Gesnerieae species (Figure 2, Table 4), and marginally in a fourth, *G. citrina* (p=0.07). No significant effect of treatment was found for mean seed mass per fruit for any of the four species, suggesting that when flowers are visited, hummingbirds effect full seed set (Figure 2, Table 4). The highest contribution of autonomous selfing to total female fertility or 1-

[(mean fruit set * mean seed mass/ fruit) of emasculated / control flowers] was for *G. reticulata* (100% and 96% for 2006 and 2007 respectively), followed by *G. cuneifolia* (56 % and 45 % for 2006 and 2007 respectively). The contribution of self-pollination was more variable across time in *G. citrina* (7% and 19% in 2006 and 2007 respectively) and *R. asperum* (0% and 18% in 2006 and 2007 respectively), and significant only for the latter species in 2007 (Figure 2).

Discussion

Pollen limitation and pollination specialization- In flowering plants, inadequate pollen transfer frequently causes reduced female fertility (Ashman et al. 2004, Knight et al. 2005). Variation in pollination success results from a variety of factors such as anthropogenic disturbance (e.g. habitat fragmentation, loss of pollinators, Wilcock and Neiland 2002), characteristics of pollinator assemblages (e.g., Gomez et al. 2007), and attributes of the plant species (e.g., Dudash 1993, Ehrlén et al. 2002). Pollination specialization is an attribute of the plant and the environment that is expected to increase the likelihood of pollen limitation (Larson and Barrett 2000), a trend documented by Knight et al. (2005) in a large survey of pollen limitation across the Angiosperms. We predicted that low pollinator visitation would make specialist Gesnerieae more vulnerable to reproductive failure than their generalists congeners (with up to ten times more pollinator visits). The results were consistent with the prediction, providing the first evidence for the association between specialization and pollen limitation within a group of closely related species.

Pollen limitation has been related to low pollinator visitation in plant species with small population sizes (e.g., Baker et al. 2000), while other studies have not found a relationship between floral visitation and pollen limitation (e.g., Duan et al. 2007). These results may reflect the importance of pollination effectiveness in addition to visitation frequency as determinants of the degree of pollen limitation experienced by plants (Aizen and Harder 2007). There are various reasons why visitation frequency appears to have a greater impact in the studied Gesnerieae species. First, differences in visitation among the different pollination systems are large, ranging from an average of one visit per flower per day for hummingbird-pollinated species to 13 visits for generalist species (Appendix II). Second, considering that average visitation can be as low as one visit per flower every three days, it is reasonable to conclude that some flowers do not receive a single visit during their receptive period (stigmas are generally receptive for two-three days in Gesnerieae). Last, generalized Gesnerieae species are visited by the same pollinators that visit specialists, in addition to other pollinator guilds. For example, specialized *Rhytidophyllum asperum* is pollinated exclusively by hummingbirds, while generalized *R. leucomallon* is pollinated by hummingbirds, bats and moths. The same species of hummingbird – the Hispaniolan Emerald – visits both *Rhytidophyllum* species in the same region of the Dominican Republic (Martén-Rodríguez et al. in press). Since having specialized flowers is generally associated with greater precision and effectiveness of pollen transfer on a per visit basis (Wilcock and Neiland 2002), it is hard to envision that the reduced fruit and seed set of tubular-flowered *R. asperum* would be due to lower quality pollen transfer by the Hispaniolan Emerald. However, having additional pollinators such as bats may increase both quantity and quality of pollen received by generalists, because bats fly long distances and are likely to transfer outcross pollen. Studies that involve measures of pollinator effectiveness will be extremely useful to assess the role of pollen quality as a determinant of the frequency and magnitude of pollen limitation.

Reproductive Assurance and Pollination Specialization- In a survey of the literature Fenster and Martén-Rodríguez (2007) found that mechanisms that facilitate autonomous self-pollination were found both in species with generalized pollination systems (e.g. *Sanguinaria canadensis*, Schemske et al. 1978), and species with specialized pollination systems (e.g. *Ophrys apifera*, Darwin 1877). The prevalence of autonomous selfing in specialized species was an unexpected finding of Fenster and Martén-Rodríguez (2007) given the long-standing notion that pollination specialization reflects selection for outcrossing and efficient pollen transfer (Darwin 1877, Zhang et al. 2005). Nevertheless, theory predicts that autonomous self-pollination should be favored if it provides reproductive assurance when pollinators are scarce (Lloyd 1992). Gesnerieae species with specialized pollination systems have significantly lower pollinator visitation frequencies than their generalist conspecifics; therefore, we expected that reproductive assurance mechanisms would be more prevalent in specialized species. This prediction was only partially supported by the data. Significant levels of autonomous self-pollination were detected only in five tubular-flowered species, all with highly specialized hummingbird pollination, while no autonomous self-pollination characterized the three species with specialized bat pollination systems (Table 3). A scarcity of autonomous selfing mechanisms in bat-pollinated species is also evident from literature surveys (Lloyd and Schoen 1992; Fenster and Martén-Rodríguez 2007). These results suggest that the morphology of flowers adapted to bat pollination may not favor breeding system traits associated with the capacity for self-pollination.

Autonomous selfing mechanisms in Gesnerieae were strictly associated with tubular flowers specialized on hummingbird pollination. Thus, one could argue that high autofertility levels are the result of the particular floral morphology and not an evolutionary response to cope with inadequate pollinator service. Narrow corollas facilitate contact between stigmas and anthers due to the proximity of stamens and stigma. However, if the ability to self-pollinate were merely a non-adaptive consequence of floral morphology, then, autonomous pollination would not confer a reproductive advantage in comparisons between emasculated and unmanipulated flowers. The significantly greater fruit set of unmanipulated flowers, detected in three out of four ornithophilous Gesnerieae (Figure 2), indicates that autonomous pollination is compensating for the low levels of vector-mediated pollen transfer exhibited by these species. Self-pollination is used as reproductive assurance by increasing fruit set and overall seed production, but not the seed set of individual flowers. The lack of differences in seed mass between emasculated and unmanipulated flowers reveals that, while hummingbirds are infrequent visitors, they are effective pollen vectors. These results along with evidence from other species support the hypothesis that plants often use autonomous selfing as a mechanism to ensure reproduction when pollinator service is low (e.g. Herlihi and Eckert 2002, Elle and Carnie 2003, Kaliz 2004, Jacquemin and Brys 2008).

Theoretical models suggest that reproductive assurance mechanisms should evolve when opportunities for outcrossing are low, contingent upon the magnitude of inbreeding depression (Lloyd 1979, Lande and Schemske 1985), and other parameters such as pollen and seed discounting (Porcher and Lande 2005). In this context the incidence of self-fertilization has been related to life history traits and growth habit (Wiens 1984, Barrett et al. 1996). For instance, high levels of inbreeding depression in long-lived woody perennials (like most bat-pollinated species) may hinder the evolution of selfing (Barrett et al. 1996). In Antillean *Gesneria*, a high dependence on self-pollination was found for two small-sized species with herbaceous or suffruticose (having a woody base) stems. Herbaceous species generally exhibit higher frequencies of self-fertilization than woody species, which has been attributed to the greater ability of small plants with shorter life cycles to purge deleterious mutations and reduce genetic loads leading to high levels of inbreeding depression (Barrett et al. 1996). Further work requiring greater sampling of species is needed to assess the role of life history traits and inbreeding depression on the evolution of reproductive assurance mechanisms in Gesnerieae species with contrasting pollination systems.

Autonomous selfing and generalized pollination systems: an escape from pollen limitation? An obvious disadvantage of specialization in pollination mutualisms is that the scarcity or absence of a pollinator jeopardizes the reproductive success of the plant. Thus, specialized plants might evolve traits that attract additional or different pollinators. Pollen limitation could be one cause for the overwhelming presence of generalized pollination systems in some pollinator depaupurate environments such as islands (Carlquist 1974, Barrett 1996). Alternatively, insular plants might evolve traits that promote autonomous self-pollination (Baker 1955, Barrett 1996). While selection to reduce inbreeding depression may favor floral phenotypes that promote outcrossing, the evolution of reproductive assurance mechanisms could also play an important role in the maintenance of floral specialization in island plants. In spite of the relative geographic proximity of the Greater Antilles to the American continent, the pollinator fauna in these islands is reduced when compared to mainland regions at similar latitudes. For example, Costa Rica has 52 species of hummingbirds (Garrigues and Dean 2007) and 10 species of nectar-feeding bats (Laval and Rodríguez 2002), while Cuba – twice the size of Costa Rica – has three species of hummingbirds (Raffaele et al. 1998) and four species of nectar-feeding bats (Koopman 1981). The less diverse pollinator fauna, combined with high levels of habitat degradation in the Antilles, probably increase the risk of reproductive failure in narrow endemic plant species. In the tribe Gesnerieae approximately 60 out of 75 species are restricted to one island, and many species have restricted distribution within islands. Thus, the presence of autonomous selfing mechanisms in some Gesnerieae safeguards against fluctuations in the composition and abundance of floral visitors, and is likely to allow the maintenance of specialized pollination systems in this insular plant taxonomic group. Likewise, having additional species of effective pollinators appears to reduce susceptibility to pollination failure. Our study suggests that pollen limitation may have been an underlying selective force in shifts from specialization to generalization and the evolution of reproductive assurance mechanisms that ensure reproduction under variable pollination environments.

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TABLE 1. Repeated measures ANOVA effects of pollination treatment (hand-pollination vs. open pollination) on fruit set and seed mass of nine species of *Gesneria* and *Rhytidophyllum* from Dominican Republic and Puerto Rico studied in 2006 and 2007.

Species	Pollination system	Fruit set		Seed mass	
		F	*P*	F	*P*
G. citrina	Hummingbird	41.63 $_{(1, 63)}$	**< 0.0001**	5.76 $_{(1, 40)}$	**0.02**
R. asperum	Hummingbird	9.07 $_{(1, 63)}$	**0.004**	8.8 $_{(1, 44)}$	**0.005**
G. cuneifolia	Hummingbird/ Self	2.02 $_{(1, 67)}$	0.160	0.67 $_{(1, 64)}$	0.415
G. reticulata	Self-pollination	0.10 $_{(1, 52)}$	0.758	0.00 $_{(1, 52)}$	0.996
G. pedunculosa	Bat	36.84 $_{(1, 53)}$	**< 0.0001**	12.07 $_{(1, 47)}$	**0.001**
G. quisqueyana	Bat	13.46 $_{(1, 43)}$	**0.0007**	2.88 $_{(1, 38)}$	0.098
G. viridiflora	Generalist	0.28 $_{(1, 75)}$	0.596	2.06 $_{(1, 67)}$	0.156
R. leucomallon	Generalist	0.64 $_{(1, 57)}$	0.426	0.34 $_{(1, 56)}$	0.564
R. vernicosum	Generalist	0.41 $_{(1, 68)}$	0.524	0.67 $_{(1, 55)}$	0.416

TABLE 2. Pollen limitation index (PLI) for nine species of *Gesneria* and *Rhytidophyllum* from Dominican Republic and Puerto Rico for 2006 and 2007. The index is calculated as 1- (Open / Pollen supplemented total seed) (Larson and Barrett 2000); total seed = fruit set *x* mean seed mass per fruit. For two *Gesneria* species with significant self-pollination, an approximate measure of pollen limitation for emasculated flowers is provided as 1- (Emasculated / Open), in brackets.

Species	Pollination system	PLI (%) [1-(E / O)]
G. citrina	Hummingbird	48
R. asperum	Hummingbird	36
G. cuneifolia	Hummingbird / Self-pollination	17 [51]
G. reticulata	Self-pollination	2 [97]
G. pedunculosa	Bat	44
G. quisqueyana	Bat	26
G. viridiflora	Generalist	12
R. leucomallon	Generalist	8
R. vernicosum	Generalist	18

TABLE 3. Potential autonomous fruit set for 15 Gesnerieae species from Puerto Rico and Dominican Republic studied in 2005-2007. The autofertility index (AFI) was calculated as the average fruit set of bagged flowers (autonomous fruit set) divided by the average fruit set of hand-pollinated outcrossed flowers. Sample sizes [n] are the number of plants used for bagging and hand pollination treatments.

Species	Pollination system	Autonomous fruit set percent (n)	AFI
G. citrina †	Hummingbird	24 (30)	0.38
G. cuneifolia †	Hummingbird	68 (15)	0.88
G. decapleura	Hummingbird *	0 (16)	0.00
G. pulverulenta	Hummingbird	13 (8)	0.19
G. reticulata †	Self-pollination	90 (28)	0.98
G. pseudobulbosa ined.	Hummingbird	4 (21)	0.07
R. asperum	Hummingbird	12 (23)	0.17
G. fruticosa	Bat	0 (10)	0.00
G. pedunculosa †	Bat	1 (22)	0.02
G. quisqueyana	Bat	0 (19)	0.00
G. ekmanii	Generalist *	0 (6)	0.00
G. viridiflora subsp. *sintenisii* †	Generalist	8 (15)	0.09
R. auriculatum	Generalist	4 (19)	0.06
R. leucomallon	Generalist	3 (19)	0.03
R. vernicosum	Generalist	2 (30)	0.03

* Inferred from floral traits.

† Autonomous fruit set from Martén-Rodríguez and Fenster 2008.

TABLE 4. ANOVA effects of reproductive assurance treatment (emasculation vs. control) and year (2006 vs. 2007) on fruit set and seed mass of four tubular flowered species of *Gesneria* and *Rhytidophyllum* from Dominican Republic and Puerto Rico.

Species	Fruit set			Seed Mass		
Source of variation	df	F	*P*	df	F	*P*
G. citrina						
Treatment	1	3.33	**0.073**	1	2.07	0.161
Year	1	0.07	0.795	1	0.03	0.853
Treatment *x* Year	1	0.59	0.445	1	0.42	0.520
Error	64			53		
R. asperum						
Treatment	1	4.29	**0.043**	1	0.13	0.715
Year	1	0.01	0.913	1	4.80	**0.034**
Treatment *x* Year	1	1.28	0.262	1	0.37	0.546
Error	57			82		
G. cuneifolia						
Treatment	1	24.30	< **.0001**	1	0.25	0.618
Year	1	0.34	0.564	1	0.10	0.752
Treatment *x* Year	1	0.02	0.902	1	0.92	0.342
Error	66			78		
G. reticulata						
Treatment (year 2007) *	1	21.89	< **.0001**		_	_
Error	32					

* Means were compared for fruit set in 2007 only; emasculated flowers set no fruit in 2006.

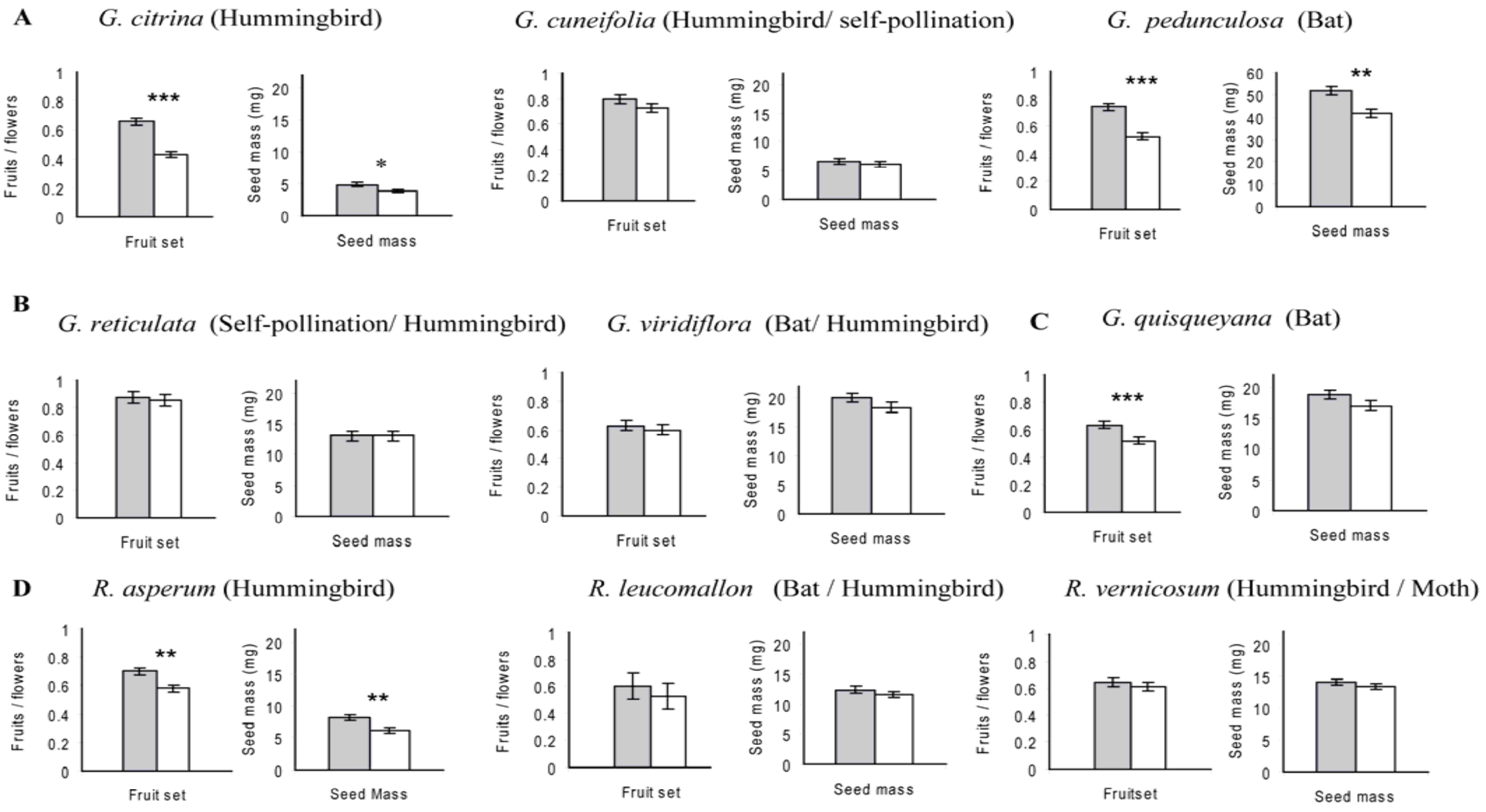

FIGURE 1. Mean percent fruit set and seed mass of pollen supplemented treatment (grey bars) and open-pollinated control (open bars) of nine species of *Gesneria* and *Rhytidophyllum* from Puerto Rico (sites A and B) and Dominican Republic (sites C and D) studied in 2006 and 2007. Error bars indicate ± SEM.

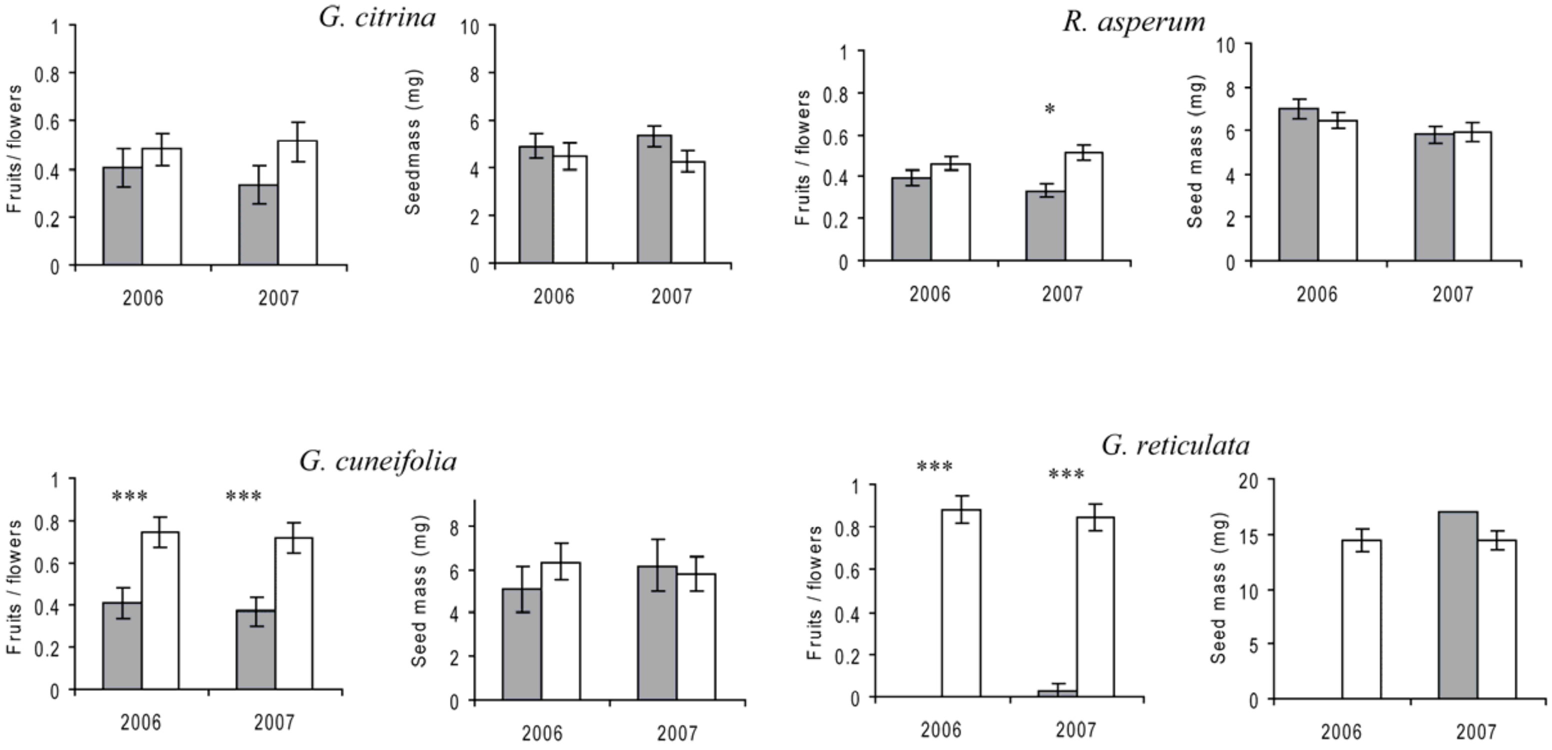

FIGURE 2. Least square mean fruit set and seed mass of emasculated (grey bars) and open pollination (open bars) treatments for four ornithophilous species of Antillean *Gesneria* and *Rhytidophyllum* studied in 2006 and 2007. Asterisks indicate a significant contribution of autonomous self-pollination to fruit set (* $p<0.05$), *** $p< 0.0005$).

Appendix I. Sites and geographic coordinates of studied populations of 15 *Gesneria* and *Rhytidophyllum* surveyed in Puerto Rico and Dominican Republic in 2006 and 2007. Site codes are provided for comparison of species used in pollen limitation experiments.

Species	Site code	Study site	Latitude N	Longitude W	Elevation m.a.s.l.	Habitat
G. citrina	A	Puerto Rico, Arecibo Observatory	18° 20'	66° 45'	300- 350	Karst, open or forested areas
G. cuneifolia	A	Puerto Rico, Arecibo Observatory	18° 20'	66° 45'	300- 350	Karst, forested areas
G.decapleura		Dominican Republic, Cordillera Central, El Convento.	18° 51'	70° 41'	1500	Cloud forest understory
G. pulerulenta		Dominican Republic, Sierra de Bahoruco, Hoyo de Pelempito	18° 05'	71° 30'	1100- 1200	Limestone, pine forest and open areas
G. reticulata	B	Puerto Rico, El Yunke National Forest, La Mina trail.	18° 18'	65° 46'	600	Rainforest, rocky substrates along river
G. pseudobulbosa ined.		Dominican Republic, Sierra de Bahoruco, road to Aceitillar	18° 06'	71° 37'	760	Karst, forested areas
R. asperum	C	Dominican Republic, Sierra de Bahoruco, Hoyo de Pelempito	18° 05'	71° 30'	1200- 1300	Limestone, pine forest and open areas
G. fruticosa		Dominican Republic, Cordillera Central, El Manaclar	19° 35'	70° 45'	1100	Rainforest understory
G. pedunculosa	A	Puerto Rico, Arecibo Observatory	18° 20'	66° 45'	300- 350	Limestone; edges and forested areas
G. quisqueyana	D	Dominican Republic, Cordillera Septentrional, Blanco Arriba, La Jíbara.	19° 30'	70° 20'	650-700	Summit of limestone hills
G. ekmanii		Dominican Republic, Sierra de Bahoruco, Cachote	18° 04'	71° 10'	1100	Cloud forest understory
G. viridiflora subsp. *sintenisii*	B	Puerto Rico, El Yunke National Forest, Mt. Britton trail	18°18'	65° 47'	700- 800	Rainforest; rocky substrates along river
R. auriculatum		Puerto Rico, Arecibo Observatory	18° 09'	66° 33'	1150	Along trails or roadsides
R. leucomallon	C	Dominican Republic, Sierra de Bahoruco, road to Aceitillar	18° 06'	71° 37'	650	Transition between dry and pine forests; along roadsides
R. vernicosum	C	Dominican Republic , Sierra de Bahoruco Nacional Park, 4Km North of Caseta 2.	18° 12'	71° 33'	1900	Pine forest and open secondary growth

Appendix II. Floral characteristics and pollinator visitation frequency for 15 species of *Gesneria* and *Rhytidophyllum* from Puerto Rico (PR) and Dominican Republic (DR) (From Martén-Rodríguez et al. in press).

Species (locale)	Corolla shape	Color	Time of dehiscence	Pollinators	No of visits/ flower/ day	
					2006	2007
G. citrina (PR)	Tubular	Yellow	Diurnal	Hummingbirds	0.4	0.3
G. cuneifolia (PR)	Tubular	Red	Diurnal	Hummingbirds	0.6	0.4
G.decapleura (DR)	Tubular	Red	Diurnal	Hummingbirds†	-	-
G. pulerulenta (DR)	Tubular	Red	Diurnal	Hummingbirds	2.7	1.7
G. reticulata (PR)	Tubular	Red	Diurnal	Hummingbirds††	0.0	0.0
G. pseudobulbosa ined. (DR)	Tubular	Orange	Diurnal	Not observed	-	-
R. asperum (DR)	Tubular	Orange	Diurnal	Hummingbirds	2.1	1.5
G. fruticosa (DR)	Campanulate	Green with purple markings	Nocturnal	Bats	1.0	1.7
G. pedunculosa (DR)	Campanulate	White - cream	Nocturnal	Bats	4.0	4.2
G. quisqueyana (PR)	Subcampanulate	White - brownish green	Nocturnal	Bats	0.9	2.1
G. ekmanii (DR)	Subcampanulate	White with shades of brown	Nocturnal	Moths†	-	-
G. viridiflora subsp. *sintenisii* (PR)	Subcampanulate	Light green with shades of brown	Nocturnal	Bats Hummingbirds Moths	1.3 3.5 3.0	3.6 2.4 3.6
R. auriculatum (PR)	Subcampanulate	Yellow with dark red markings	Nocturnal	Bats Hummingbirds	1.7 1.0	- -

R. leucomallon (DR)	Subcampanulate	Light yellow	Nocturnal	Bats	-	4.7
				Hummingbirds	-	6.0
				Moths	-	1.3
				Flies and bees	-	1.3
R. vernicosum (DR)	Subcampanulate	Yellow with red markings	Diurnal and nocturnal	Hummingbirds	7.1	10.4
				Moths	1.5	5.1
				Flies and bees	1.7	0.5

† Anecdotal observations of pollinators, visitation rates not quantified.

†† Only one hummingbird visit observed in 40 hours of observation over 3 years.

CHAPTER 4

Phylogeny and floral evolution of an Antillean plant radiation: Insights from ecology, morphology and molecular data.

Co-authored with Charles B. Fenster, Laurence E. Skog and Elizabeth A. Zimmer

Abstract

The tribe Gesnerieae (family Gesneriaceae) represents a plant radiation from the Antilles that encompasses great variation in floral phenotypes (e.g. tubular, campanulate, rotate flowers) and breeding systems (e.g. different states of dichogamy and levels of autogamy). This study examines the evolutionary history of floral characters and breeding systems in relation to pollination system transitions in Gesnerieae. Traditional schemes of generic and sectional classification are also evaluated in light of phylogeny and pollination biology. Phylogeny reconstruction used two nuclear DNA regions (ITS and *GCYC*) and a morphological data set of 37 characters. Maximum parsimony, Maximum Likelihood and Bayesian analyses were performed on each data set separately and on the combined data sets. An assessment of floral character evolution was conducted for floral traits, breeding systems, and pollination systems using the total evidence phylogenetic hypothesis. Analyses of molecular and morphological data sets using different optimality criteria resulted in similar topologies. All analyses indicate the genus *Gesneria* is paraphyletic, including *Rhytidophyllum* and perhaps *Pheidonocarpa*. The position of the small genus *Bellonia* (with rotate flowers) appears to be basal. Most parsimonious floral character reconstructions indicate two origins for species with campanulate flowers (specialized on bat pollination) and four origins for subcampanulate flowers (generalized pollination by bats, moths and hummingbirds). The acquisition of bat pollination involves changes in corolla shape, floral phenology and nectar production, while maintenance of hummingbird pollination in the generalist is associated with corolla constriction. Pollination by bees is rare, occurring in *Bellonia* and perhaps a few other unstudied species. Autonomous self-pollination evolved three times in lineages with ornithophilous flowers. This study provides evidence for transitions between specialized pollination systems, and from specialized to generalized systems associated with consistent transitions in floral characters. The evolution of reproductive assurance mechanisms as well as transitions from specialized hummingbird pollination to alternative pollination systems (bats and generalists) may be related to the low frequency of hummingbird visitation to Gesnerieae flowers in the Antilles. The maintenance and diversification of ornithophilous lineages may be facilitated by the evolution of autonomous self-pollination mechanisms. These results support the notion that pollinator depauperate faunas on islands create selective environments that favor the evolution of generalization and autonomous breeding systems.

Key words: Antilles, floral evolution, *GCYC*, Gesneriaceae, islands, ITS, phylogeny, pollination systems.

Introduction

Ocean archipelagoes provide natural laboratories for studying the processes underlying diversification and adaptation (Emerson, 2002). For instance, studies of insular plant groups have shed light on ecological factors promoting the evolution of morphological and anatomical traits (Hawaiian silverswords, Carlquist, 1974; Baldwin, 2006), plant breeding systems (e.g. Hawaiian *Schiedea*, Sakai et al., 2006) and the specificity of biotic interactions (Canarian Psyllids and Legumes, Percy, 2003). Two common patterns that emerge from studies of plants in ocean archipelagoes are the convergent evolution of traits in response to similar environments on different islands (e.g., Hawaiian Lobeliads, Montgomery and Givnish, 2008), and the evolution of reproductive assurance mechanisms associated with scarcity of mates or pollinators (Carlquist, 1974). This study examines the patterns of pollination and breeding system evolution in an Antillean plant radiation in order to understand the factors that promote floral diversification and adaptation.

Plant-pollinator interactions have great potential to influence floral evolution in ocean archipelagoes (Barrett, 1996). Similar pollinator faunas on different islands may generate similar selective environments on floral traits resulting in convergent evolution of floral phenotypes. Furthermore, the depauperate pollinator faunas of many islands may impose selective pressures favoring changes in plant-pollinator associations, as well as the evolution of wind pollination and autonomous breeding systems (e.g. community survey of Galapagos angiosperms; McMullen, 1987). Thus, for insular plant groups, transitions from specialized ancestral floral morphologies to phenotypes that promote pollination by a wide range of floral visitors (i.e.

evolutionary generalization) might be expected, particularly when the original pollinators are absent or scarce. Unspecialized flowers with dull colors and open access rewards in fact prevail on remote oceanic islands (Carlquist, 1974; Barrett, 1996). However, specialized floral phenotypes are also present in some insular plant groups (Antillean Gesneriads, Skog, 1976; Hawaiian Lobeliads, Givnish et al., 1995, Hawaiian mints, Lindqvist and Albert, 2002). While the origin of specialized pollination systems on islands may be associated with the presence of novel or more effective floral visitors, the maintenance of specialization in pollinator depauperate environments may require the possession of breeding mechanisms that provide reproductive assurance (Jain, 1976).

Comparative approaches provide significant insights into the role of pollinator-mediated selection on floral diversification and breeding system evolution (Armbruster, 1992; McDade, 1992). For instance, the independent evolution of similar floral traits within a phylogeny, consistently associated with shifts to a common pollination mode, provides indirect evidence of adaptation. Recent studies show that pollinator shifts are common in the evolutionary history of many angiosperms, revealing frequent convergence and high lability of floral characters subject to pollinator-mediated selection (e.g. Armbruster, 1992; Weller and Sakai, 1999; Perret et al., 2001; Kay et al., 2005; Tripp and Manos, 2008; reviewed in Fenster et al., 2004). Yet, surprisingly little information is available on the patterns of pollination system evolution based on phylogenetic studies of insular plant radiations.

Although a considerable number of plant phylogenies are available for island plants, our understanding of phylogenetic patterns of pollination system evolution is limited by the number of pollination ecology studies available for medium or large-sized plant taxonomic groups. For example the Caribbean islands, considered one of the biodiversity hotspots of the planet (Myers et al., 2000), provides numerous examples of plant radiations (e.g. Tribe Gesnerieae, Skog 1976; *Exostema*-Rubiaceae, McDowell and Bremmer, 1998; Santiago-Valentin and Olmstead, 2004). However, research on plant reproductive biology on these islands has focused primarily on individual species (e.g. Temeles and Kress 2003; Gonzáles-Díaz and Ackerman, 1998), or plant communities (e.g. KodricBrown et al., 1984; Dalsgaard et al., 2008). To our knowledge, only two plant groups have been studied extensively for their pollination and breeding system ecology, the Caribbean *Passiflora* (Kay, 2003) and the Antillean tribe Gesnerieae (Martén-Rodríguez and Fenster, 2008, Martén-Rodríguez et al., in press). Only the latter represents a monophyletic Caribbean radiation. The Gesnerieae comprises approximately 75 species with a distribution centered in the Greater Antilles (Zimmer et al., 2002). Recent field studies have documented both specialized (e.g. hummingbird, bat, bee) and generalized pollination systems (involving hummingbirds, insects and bats), as well as autonomous self-pollination mechanisms in some members of the tribe (Martén-Rodríguez and Fenster 2008, Martén-Rodríguez et al., in press). The association of floral phenotypes with particular pollination systems suggests that pollinators have had a significant influence on the diversification of the tribe. Due to its insular geographic distribution, monophyly, great floral diversity and availability of comprehensive reproductive biology information, the Gesnerieae makes an exceptional study system to examine patterns of floral diversification within a phylogenetic context.

While phylogenetic studies provide an important framework for understanding patterns of pollination and breeding system evolution, the study of plant reproductive attributes can provide important insights into the utility of floral traits that are traditionally used in taxonomic classification (Anderson et al., 2002). Floral characteristics that are subject to pollinator-mediated selection may provide misleading results if used as primary characters to establish classification systems because they may be subject to frequent convergence or parallelism. The evolution of breeding system characteristics in response to reduced pollinator service may also be responsible for convergent patterns in floral morphology (Culley and Klooster 2007). Thus, the use of reproductive traits in taxonomic classification of plant adaptive radiations must be considered carefully in light of their evolutionary history and pollination ecology.

Here, we use a phylogenetic approach to examine the evolutionary patterns of floral specialization and generalization and the evolution of reproductive assurance mechanisms in the tribe Gesnerieae, in order to increase our general understanding of the evolution of pollination and breeding system traits. We generated phylogenies using molecular and morphological data and asked the following questions: (1) are pollination systems evolutionary labile in Gesnerieae? (2) is there evidence for convergent or parallel evolution of floral characteristics in response to changes in pollination systems? (3) does generalization in pollination systems evolve from specialization? (4) is the evolution of reproductive assurance mechanisms associated with particular pollination system? We also examine the current generic and sectional classification of the Gesnerieae and assess the utility of floral characters for taxonomic classification.

Materials and methods

Study System- The tribe Gesnerieae (family: Gesneriaceae) encompasses four genera and approximately 75 species, most of which are restricted to the Antillean islands. Three species occur in northern South America, the putative center of origin for the tribe (Skog, 1976; Wiehler, 1983). The tribe has been characterized by having an alternate leaf arrangement in most species, a basal chromosome number of $n=14$, and petiole vascular bundles forming a ring (Wiehler, 1983). According to subfamily-level studies, the Gesnerieae is a monophyletic tribe (Smith, 1996; Zimmer et al., 2002; Roalson et al., 2005) consisting of four genera: (1) *Gesneria*- with 53 species displaying great variation in growth form and overall morphology, (2) *Rhytidophyllum*-with 19 species of shrubs characterized by having bullulate leaf surfaces and subcampanulate yellow, often dark red spotted corollas (although tubular red corollas are found in a few species), (3) *Pheidonocarpa*- a monotypic genus with two subspecies narrowly distributed in Cuba and Jamaica, has opposite or decussate leaves, and tubular ornithophilous flowers, (4) *Bellonia*- with two species of small shrubs characterized by having opposite leaves and rotate, white flowers; occurring in Cuba and Hispaniola. This genus was originally classified within the tribe Gloxinieae; however, recent higher order molecular phylogenies give strong support for *Bellonia* as a member of the tribe Gesnerieae (Smith et al., 2004a; Roalson et al., 2005).

The taxonomic delimitation of the genus *Rhytidophyllum* has been subject of controversy. Although most *Rhytidophyllum* species share a set of morphological features that distinguishes them from *Gesneria* (e.g. bullulate leaves, subcampanulate flowers, stamens distinctly adnate to the base of the corolla, isolated stomata raised on domes, and undulate cell margins), there are species that lack one or more of these character states (e.g. plane leaf surfaces in *R. petiolare*, tubular flowers in *R. asperum*), and species that have mixed traits of both genera (e.g. *Gesneria rupincola*, *Gesneria earli*; Skog, 1976; Wiehler, 1983). The occurrence of these different combinations of character states raises the question whether *Gesneria* and *Rhyitdophyllum* should be considered separate genera (see Wiehler, 1983).

The pollination systems of 19 Gesnerieae species from the Greater Antilles and St. Lucia were characterized in earlier studies by conducting pollinator observations from 2003 to 2007 for a total of 602 observation hours (Martén-Rodríguez and Fenster, 2008; Martén-Rodríguez et al., in press). Floral phenotypes in Gesnerieae are distinctly associated with particular pollination systems as follows: (1) tubular, brightly colored, diurnal flowers have specialized hummingbird pollination, (2) campanulate white or green flowers with nocturnal anther dehiscence are primarily bat-pollinated, (3) subcampanulate flowers with nocturnal anther dehiscence and varied in color have generalized pollination systems (including bat, moth and hummingbird pollination). Additional floral phenotypes include white tubular or funnel-shaped diurnal flowers (possibly bee-pollinated), and rotate flowers, characteristic of *Bellonia* and associated with buzz pollination by large bees. The principal floral phenotypes are shown in Table 1.

Breeding system variation in the tribe Gesnerieae includes variation in floral traits associated with timing of anther dehiscence and stigma receptivity, and in the frequency of self-pollination. The methods used to determine the frequency of autonomous self-pollination of 15 Gesnerieae species are described in Chapter 3 (Martén-Rodríguez and Fenster, unpublished manuscript). Only species with tubular flowers have any significant levels of autonomous self-pollination (> 10 % fruit set upon self-pollination), although there is great variation among species (Chapter 3). Campanulate, subcampanulate and rotate-flowered species have levels of autonomous self-pollination ranging between 0 and 9%. The autofertility index proposed by Lloyd (1992) and estimated as: AI = autonomous fruit set/open pollinated fruit set, will be used here as the measure of the level of autonomous self-pollination to be mapped onto the phylogeny. The maturation time of reproductive organs was recorded to determine the level of dichogamy (protogyny, protandry or adichogamy) while conducting pollinator observations; character states listed in morphological data matrix (Appendices I and II).

Taxon sampling- The ingroup included thirty-five species of the tribe Gesnerieae, including samples of all four genera. Thirty-one species were collected in the field in Dominican Republic, Jamaica, Puerto Rico and St. Lucia. Voucher specimens were deposited at Smithsonian National Museum of Natural History Herbarium (US), Herbario del Jardín Botánico Nacional, Santo Domingo, Dominican Republic (JBSD), and Institute of Jamaica (IJ) and leaf and flower tissues were stored in silica gel. Sequences of four Cuban Gesnerieae and three outgroup species (*Gloxinia erinoides*, *Monopyle macrocarpa* and *Kohleria hirsuta*) were obtained from Genbank

(Appendix III). Outgroups were selected exclusively from the tribe Gloxinieae because previous studies have presented strong support for the Gloxinieae as sister tribe to the Gesnerieae (Smith et al., 2004a Zimmer et al., 2002). Although our data set is missing most of the endemic species from Cuba, it includes good sampling for all other islands, and more importantly it includes a fair representation of floral variation consistent with the overall proportions of floral phenotypes for the tribe.

DNA sequencing- Genomic DNA was extracted from silica-dried leaf tissue using the Qiagen DNeasy DNA isolation kit (Qiagen Valencia, California, USA); tissue samples were first macerated in liquid nitrogen with a mortar and pestle. Polymerase chain reactions (PCR) amplifications were performed for nuclear ribosomal ITS and for the nuclear developmental gene G CYCLOIDEA (*GCYC*). Templates for the *ITS* region were obtained using primers ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'; White et al., 1990) and ITS5 HP (5'-GGA AGG AGA AGT CGT AAC AAG G-3'; Suh et al., 1993). Amplification reactions for ITS were carried out for 30 cycles (denaturation at 94 ºC, annealing at 55 ºC and elongation at 72 ºC, followed by 6 minutes at 72 ºC) using TAQ polymerase (Promega, Madison, Wisconsin, USA). PCR products were checked by electrophoresis in 1.0% agarose gels in 1X TBE (pH 8.3) buffer and staining with ethidium bromide. Single products were cleaned using the PEG (polyethylene glycol) precipitation procedure (Johnson and Soltis 1995). Direct cycle sequencing of purified templates used ABI Prism BigDye Terminator Cycle Sequencing, with primers ITS5 HP, ITS 4 and two internal primers, ITS2 and ITS3 (White et al., 1990).

Amplification of *GCYC* templates followed the procedure described above but with an annealing temperature of 50 ºC and using Biolase polymerase (Bioline, UK). Primers for amplification of *GCYC* were cycFS1 (5'- MTGGTTSCTCACTARATC-3'; Smith et al., 2004b and cycR (5'-ATG AAT TTG TGC TGA TCC AAA ATG; Moller et al., 1999). Gene duplication events in GCYC have occurred in Old World lineages of Gesneriaceae; however, only one copy of *GCYC* is present in the New World Gesnerioidae (Smith et al., 2004b Wang et al., 2004), the clade that contains the study tribe Gesnerieae. To test the utility of chloroplast regions for phylogenetic reconstruction of the Gesnerieae, amplifications were conducted for chloroplast spacers trnL-F and psbA-trnH in a subset of species (n=25 and n=15 respectively). DNA sequence variation was low (1 % for trnL-F and 3.9% for psbA-trnH, with few phylogenetically informative characters); therefore plastid regions were not further investigated.

Sequences for both DNA strands of ITS and *GCYC* were assembled and edited using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Manual alignment of DNA sequences was performed in SeAl v2.0a11 (Rambaut, 1996). Sequences of the ITS region were truncated to include only ITS1, 5.8S and ITS2, by comparison with sequences of various *Gesneria* and *Rhytidophyllum* species previously published (Zimmer et al., 2002).

Morphological data matrix- Character states were scored for 34 Gesnerieae species and three outgroups by examination of herbarium specimens (at US and JBSD), live plants and the literature (Skog, 1976, 1978; Wiehler, 1970; 1983; Kriebel Haehner, 2006; Xu and Skog, unpublished manuscript). A total of 37 morphological characters were used: 18 characters were associated with vegetative morphology, 16 with inflorescence and flower traits, one with fruit morphology, and one of chromosome number (Appendix I.). Chromosome number, three characters of leaf epidermis morphology (presence of stomatal domes, stomatal clusters, cell sinuation), and one anatomical character (petiole vasculature), were obtained entirely from the literature (Wiehler 1970, 1983, Skog 1976). Character states for these four traits were not available for all species, but the available data includes representatives of all genera. Some characters that were initially scored were excluded from the analysis due to the difficulty of unambiguously determining character states (e.g. fruit shape, anther shape, leaf apex shape).

Phylogenetic analyses- Maximum Parsimony analyses for ITS, *GCYC*, and the combined data set were performed in PAUP 4.0b10 (Swofford, 2002). Heuristic searches were conducted using 10,000 random addition cycles (ACCTRAN; TBR branch swapping). Branch support was estimated using 500 heuristic bootstrap replicates with 10 random addition cycles, and TBR branch swapping. Additional searches for the most parsimonious tree were performed in WinClada (Nixon, 2002) and NONA (Goloboff, 1999), yielding the same results.

The incongruence length difference (ILD; Farris et al., 1994) test was performed to assess topological congruence between trees obtained from different datasets (ITS, *GCYC*, morphology, and the two DNA regions combined). The tests were performed in WinClada and NONA (100 replicates, 10 of random addition sequences and TBR branch swapping). Incongruence was also examined by visually comparing tree topologies.

Maximum likelihood analyses were conducted in PAUP 4.0b10 (Swofford, 2002). Models of DNA sequence evolution were estimated in ModelTest version 3.7 (Posada and Crandall, 1998); parameters first estimated using a neighbor Joining tree and JC69 model of evolution. An ML search with 10 random addition replicates was run and the resulting tree was used to re-estimate parameters. This procedure was repeated until the same model of evolution was obtained two consecutive times. Heuristic searches for the best tree under the ML optimality criterion were conducted in PAUP 4.0b10 (Swofford, 2002), using 500 random addition sequence replicates, TBR branch swapping and model parameters obtained from ModelTest for each data set (Table 1). Branch support was estimated by performing 500 heuristic bootstrap replicates (with 5 random addition cycles) in GARLI (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006) using Grid computing (Cummings and Huskamp, 2005) through The Lattice Project (Bazinet and Cummings, in press).

Bayesian analyses were conducted in MrBayes V3.04 (Huelsenbeck and Ronquis, 2001, Ronquist and Huelsenbeck 2003) for the concatenated sequences of the two DNA regions. 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. A parameter for a proportion of invariant sites was added to the model specified for ITS as previously determined in ModelTest (Posada and Crandall, 1998). The Markov chain Monte Carlo search was run on four chains for 10 million generations with sampling every

1000 generations. The first 4000 generations were discarded as 'burn-in" after inspection of likelihood plots.

Analyses of morphological and combined data- Maximum parsimony analyses of the morphological dataset of 37 species were conducted in WinClada (Nixon, 2002) and NONA (Goloboff, 1999), using a heuristic search (1000 random addition cycles, TBR+TBR branch swapping). Branch support was estimated using 500 bootstrap replicates with 10 random addition sequences. Two species not included in the combined analyses due to missing data for *GCYC* (*G. rupincola* and *R. exertum)* were included in analyses of morphological data and ITS only, because they are the only available endemic species from Cuba, an island that is underrepresented in our sampling. Furthermore, *G. rupincola* has morphological characteristics that are intermediate between *Gesneria* and *Rhytidophyllum*; thus, the inclusion of this species is important for the assessment of the monophyly of the two genera.

Maximum Parsimony analyses of the combined molecular and morphological dataset (total evidence for 35 species) were run in WinClada (Nixon, 2002) and NONA (Goloboff, 1999), using the specifications described above for parsimony heuristic searches. Additionally, a Bayesian analysis of the total evidence data set was performed in MrBayes V3.04 (Ronquist and Huelsenbeck, 2003). The search used the standard model for morphological data as implemented in MR Bayes, and nucleotide substitution models and other search terms were as described above for Bayesian searches.

Character mapping- Floral characters and pollination systems were mapped onto the total evidence tree using Mesquite (Maddison and Maddison, 2004). The following characters were mapped: 1. Corolla shape: (0) tubular, (1) campanulate, (2) subcampanulate, (3) funnelform, (4) rotate; 2. Timing of anther dehiscence and nectar production: (0): diurnal, (1) nocturnal. These two traits were perfectly correlated in all of the species surveyed, therefore they are jointly mapped onto the phylogeny; 3. Corolla color: (0) red, (1) yellow with red spots, (3) yellow (4) white and (5) green; 4. Pollination system: (0) hummingbird, (1) bat, (2) moth, (3) insect generalist; 5. Dichogamy: (0) adichogamous, (1) protogynous, (2) protandrous); 6. Reproductive assurance (index of autonomous self-pollination-IA): (0) potential RA< 10%, (1) potential RA> 10%. Geographic distribution was also mapped on the phylogeny, but because only a small number of Cuban species are included in this analysis, mapping is only a preliminary overview of geographic distribution and dispersal patterns in Antillean Gesnerieae.

Of the abovementioned characters, corolla shape, corolla color (scored as the presence/absence of red and yellow pigments) and dichogamy (scored as protogyny, protandry and absence of dichogamy) were included in the matrix used for phylogenetic reconstruction. The use of trees that are at least partly based on morphological data to reconstruct the evolutionary history of morphological characters has been the subject of contention (Baker et al., 1998). However, the percent of characters that were used in both phylogenetic reconstruction and mapping was only 0.03% (4 out of 154 parsimony informative characters). We checked for the effect these characters had on tree topology by removing each character at a time and conducting parsimony searches on both the morphological and total evidence data sets in WinClada (Nixon, 2002) and NONA (Goloboff, 1999). The exclusion of the characters did not affect tree topology; therefore, we used all the available evidence. While there is some uncertainty in the reconstruction of the Gesnerieae phylogeny due to low DNA sequence divergence and limited morphological variation, the correspondence of the results obtained by analyzing different types of data (DNA and morphology), as well as the consistency of different methods of inference, suggest a total evidence approach is appropriate. The total evidence phylogeny provides a more resolved and robust estimate of the phylogenetic relationships and the best available phylogenetic hypothesis for making inferences of character evolution.

Results

Maximum Parsimony analyses of molecular data- The length of the aligned sequences and summary results for parsimony analyses performed on the three different data sets (ITS, *GCYC*, the combined DNA regions) are shown in Table 1. The analysis of the combined DNA regions (Fig. 2) provides a better resolved topology than ITS or *GCYC* alone (topology similar to ML trees described below). The ILD test indicated significant incongruence between ITS and *GCYC* tree topologies (*P*=0.001). Visual examination of tree topologies showed that disagreement occurs in the alternative placement of four species on the *GCYC* tree: *G.citrina* and *Rhytidophyllum sp. nov.,* nested within the *G. ventricosa* clade, *Bellonia aspera*, nested within the *G. humilis* clade and *G. clandestina* forming a clade with *G. ekmanii*. All these clades have ≤ 65% bootstrap support (although posterior probabilities are higher).

Maximum Likelihood and Bayesian analyses of molecular data- Nucleotide substitution models selected by ModelTest were GTR+Γ for the ITS, TVM for *GCYC* and GTR+I + Γ for the combined DNA regions. Heuristic searches for Maximum Likelihood found four trees for the combined dataset (-ln= 4197.06944; Fig. 3), one tree for ITS alone (-ln=2590.58461, Appendix IV), and five trees for *GCYC* (-ln= 1402.98759, Appendix V); where more than one tree with the same likelihood was obtained, strict consensus trees were calculated. The trees obtained under Bayesian inference were almost identical to the ML trees; therefore, the ML topologies are shown with associated bootstrap (for ML analyses) and posterior probability (for Bayesian analyses) measures of branch support. Parsimony and likelihood-based methods of phylogenetic inference produced the same results, except that one clade obtained slightly greater resolution of the Bayesian trees. Branch support values are also consistent among methods, although posterior probabilities were higher at certain nodes as has been seen in many other studies (Erixon et al. 2003, e.g., Ekenas et al. 2007).

Phylogenetic analysis of morphological and combined datasets- The analysis of the morphological dataset resulted in 32 most parsimonious trees (Length=160, CI=0.344, RI=0.717, RC=0.246). The strict consensus topology is presented in Fig. 4. A parsimony search (tree not shown) was conducted on the morphological data set excluding the two Cuban endemic species in order to have comparable morphological and molecular results and assess combinability. The topologies obtained from analysis of the molecular and morphological datasets are similar, as major clades are recovered in both analyses. The ILD test indicated significant incongruence between morphological and molecular data sets ($p=0.01$). However, visual inspection reveals three points of disagreement, all involving clades that have low branch support (< 50% bootstrap). The only point of incongruence supported by high bootstrap values was found in the placement of *Bellonia,* which morphological data place within the tribe Gloxinieae, while molecular data support its placement within the tribe Gesnerieae. However, because *Bellonia* is basal, the conflict does not affect relationships among other ingroup members. Based on these considerations, the results for total evidence phylogenetic analyses are presented. Parsimony and Bayesian analysis of the total evidence data set yielded similar results, except for an observed slightly greater resolution of the Bayesian topology (Fig. 5). Summary statistics for the parsimony search are shown in Table 1.

Evolution of floral characters and pollination systems- When considering character reconstructions we refer primarily to the clade of Gesnerieae that comprises all species currently classified in the genera *Gesneria*, *Rhytidophyllum* and *Pheidonocarpa,* to the exclusion of *Bellonia.* The latter genus, which has a basal placement, exhibits a unique specialized floral phenotype associated with buzz pollination by bees. Parsimony mapping of floral characters onto the Bayesian total evidence phylogeny indicates various transitions in floral traits and pollination system. Mapping of pollination systems reveals that hummingbird pollination is ancestral with two origins of bat pollination and four origins of generalized pollination systems. Tubular flowers (hummingbird-pollinated) are ancestral in the clade that comprises all Gesnerieae to the exclusion of basal *Bellonia*. Campanulate and subcampanulate flowers are independently derived from tubular flowers (Fig. 6).

Campanulate flowers originate twice and are associated with documented shifts to bat pollination, while subcampanulate flowers originate four times, three associated with documented shifts to generalized pollination (by hummingbirds, bats and moths), and one, *G. ekmanii*, supported only by anecdotal observations of moth visitors. Hummingbird pollination is inferred based on our field studies for 19 Gesnerieae where hummingbirds visited all species, while being effective pollinators of tubular and subcampanulate flowers only (Martén-Rodríguez et al., in press). Nocturnal anther dehiscence and nectar production evolved 6 times in association with acquisition of nocturnal pollinators, primarily bats (Fig. 7).

The most parsimonious character reconstruction of flower color indicates red corollas are ancestral with various transitions to different corolla colors and color patterns (yellow, green, white, and yellow corollas with dark red spots or markings; Fig. 8). Complete loss of corolla anthocyanines is a derived condition not consistently associated with pollination systems. While white or green corollas often characterize bat-pollinated flowers, there are yellow and white-flowered lineages with diurnal flowers (*G. citrina,* hummingbird-pollinated; *Bellonia aspera,* bee-pollinated). Two species with light-colored flowers have unknown anthesis and pollination systems (*G. humilis*, *R. petiolare*).

Stamen pubescence was another character observed to co-vary with the pollination system. The presence of a tuft of hairs at the base of the stamens evolved four times in Gesnerieae, all associated with the acquisition of bat pollinators (Fig. 9). The degree of stamen adnation, a character hypothesized to be an adaptation to bat pollination in *Rhytidophyllum* (Skog, 1976) did not co-vary with the pollination system.

The ability to set seed autonomously has evolved three times, as indicated by mapping of the index of reproductive assurance; all three origins are associated with hummingbird pollination and protogynous lineages (Fig. 10). Mapping of dichogamy shows that protandry (i.e. male organs develop first) has arisen twice from protogyny (where female organs develop first). Lack of dichogamy (the temporal separation of sexual phases) arose once in *Gesneria reticulata*, and it is also the character state for basal *Bellonia* (Fig. 10). Shifts in dichogamy states are not associated with any particular pollination system.

Mapping of geographic distribution is presented to document dispersal between islands (Fig. 11). Every well supported clade in the phylogeny has species from more than one island and some species have populations across multiple islands. Further analysis of biogeography is not attempted here due to the low representation of Cuban taxa and the missing South American species.

Discussion

Organisms on islands experience unique selective pressures 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 and Kelly, 1993), suggesting transitions from specialization to generalization have been common in the evolutionary history of island plants. However, transitions from generalized to specialized or between specialized pollination systems are the most frequent in studies of floral evolution conducted primarily on mainland plant taxonomic groups (reviewed in Weller and Sakai, 1999; Fenster et al., 2004; Tripp and Mannos, 2008). This study provides the first evidence supported by pollination ecology data for evolutionary transitions from specialized to generalized pollination systems in a monophyletic plant group from the Antillean 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. We first evaluate the traditional taxonomic classification in Gesnerieae in light of the phylogeny and briefly discuss preliminary biogeographic patterns.

Gesnerieae classification- The phylogenetic hypothesis based on a total evidence approach presented here supports three major findings. First, the genus *Bellonia* is part of the Gesnerieae as recently suggested by higher order phylogenies based on molecular data (Smith et al., 2004a, Roalson et al., 2005). Analysis of molecular data as well as the total evidence phylogeny suggests *Bellonia* has a basal placement within the tribe (Figs. 2, 3, 5). Morphological data alone support the traditional classification for *Bellonia* within the tribe Gloxinieae (Fig. 4). Various key characters present in most Gloxinieae, but absent or rare in Gesnerieae indicate a basal placement for *Bellonia*, e.g. chromosome number n=13, vascular bundle a crescent, opposite leaves (Wiehler, 1983). Floral traits also disagree with the placement of *Bellonia* within Gesnerieae; *Bellonia* has rotate flowers, no nectary, and poricidal anther dehiscence, the latter two being derived traits associated with buzz pollination by large bees (Buchmann, 1983; Larson and Barrett, 1999). While this floral phenotype appears to have evolved various times in the Gloxinieae (Roalson et al., 2005), it is not present in other extant Gesnerieae species. At this point, the available data do not allow clear determination of the closest relative to *Bellonia*. DNA sequencing of two unsampled South American *Gesneria* might help improve resolution at the root of the phylogeny, given that the ancestor of the Gesnerieae was most likely of South American origin.

A second finding that has implications for taxonomic classification is the paraphyly of the genus *Gesneria*. Four highly supported clades in the total evidence phylogeny contain species that are classified within the genus *Gesneria;* monophyletic *Rhytidophyllum* is nested within one of these clades (Fig. 5). These results correspond with the original classification scheme by Linnaeus (1753), who treated Antillean Gesnerieae as a part of a single genus. Various other classification systems have been put forth since then (reviewed in Wiehler, 1983), but the most recent taxonomic treatment considers *Gesneria* and *Rhytidophyllum* as separate genera (Skog, 1976). However, the main characters used to distinguish these genera are inconsistent, as there are various species with intermediate states (e.g. degree of stamen adnation to corolla; Skog, 1976; Wiehler, 1983). Of the traits included in this study only two are synapomorphies for the *Rhytidophyllum* clade: deep cell wall undulation and areolate leaf adaxial surfaces (except in *R. petiolare*). Thus, most evidence, both molecular and morphological, support a classification of single generic unit rather than two separate genera, as proposed earlier by Wiehler (1983).

Third and last, when the sectional classification for the genus *Gesneria* is examined in light of the phylogeny, only two clades are entirely consistent with the traditional sections proposed by Skog (1976): section Duchartrea (*G. viridiflora sintenisii* and *G. quisqueyana*), and section Physcophyllon (Fig. 5) In his treatment of *Gesneria* Skog (1976) lumps the taxa in section Duchartrea into one species with various subspecies, *G. viridiflora,*. However, we consider *Gesneria quisqueyana* from Hispaniola a separate species, as was originally described, because it differs from subspecies of *G. viridiflora* from Hispaniola and other islands in the timing of flowering and has a unique floral phenology. *Gesneria quisqueyana*, a protogynous bat-pollinated species, excludes diurnal floral visitors by actively closing corollas during the day, while all other Gesnerieae keep their flowers open for two-three days (Martén-Rodríguez et al., in press). The second supported clade within *Gesneria*, Section Physcophyllon comprises *Gesneria* species with roseate growth habit, bullate leaves and ornithophilous flowers; these plants occur primarily mostly in karst regions, forming large aggregations on rock walls and cave mouths, thus sharing morphological as well as ecological traits. The remaining major sections into which the genus *Gesneria* was divided (Skog, 1976) were not supported as monophyletic clades and two small sections from Cuba and Jamaica were not included in this study. A reassessment of the sectional classification is needed in future studies.

A discussion of biogeographic patterns of distribution and dispersal is not attempted here because sampling of the Cuban Gesnerieae is highly incomplete. However, two points are worth mentioning; first, the presence of species from various islands is supported in all clades of the phylogeny (Fig. 11), suggesting that dispersal events have been relatively frequent. Because Gesnerieae seeds are minute and seed pods contain hundreds to thousands of seeds, dispersal by wind is likely the mode of long-distance dispersal. However, geological history accounts suggest that eastern Cuba, northern Hispaniola and Puerto Rico were connected until the early to mid Miocene (20-20 Ma), by which time all the greater Antilles were above sea level (Iturralde Vinent and MacPhee, 1999; Santiago-Valentin and Olmstead 2004). Thus, some dispersal and diversification could have occurred while the land masses were still connected. The second point concerns the paucity of Gesnerieae species in the Lesser Antilles; only two species occur in this archipelago, one of which, *G. ventricosa*, has different subspecies on different islands. The low levels of diversification of Gesnerieae in the Lesser Antilles correspond to those of some other plant taxa in the region (e.g. *Exostema,* Mc Dowell and Bremer, 1998; Lythraceae, Graham 2003) This may have to do with the smaller area and younger age of this archipelago (Santiago-Valentin and Olmstead 2004), as well as the direction of trade wind currents.

Floral evolution- Pollinators have traditionally been considered the most important agents of selection underlying the widespread patterns of floral convergence exhibited by the angiosperms (Stebbins, 1970; Fenster et al., 2004). A number of studies have supported this notion by documenting the evolution of floral characteristics associated with pollination system transitions within a phylogenetic context (reviewed in Fenster et al., 2004). These studies demonstrate that transitions are common between different specialized pollination systems (e.g. Armbruster, 1988, 1993; Bruneau, 1997; Johnson et al., 1998; Goldblatt et al., 2002; Kay et al., 2005; Tripp and Mannos, 2008), or from generalized to specialized pollination systems (e.g. Luckow and Hopkins, 1995; Beardsly et al., 2003). We define specialization as a condition where flowers have adapted to a particular functional group of pollinators (sensu Fenster et al., 2004). Pollinator functional groups are here defined on the basis of taxonomic affinity as well as similarity in behavior while feeding at flowers (e.g. bats or hummingbirds). The results of this study provide evidence for a less common evolutionary trend: the repeated origin of floral traits associated with generalized pollination derived from ornithophily. Bat pollination also evolves from ornithophily. Thus, in Antillean Gesnerieae pollination system transitions occur by either shifting to alternative pollinators (bats) or by adding different functional groups of pollinators (bats and or moths). Inclusion of the putative basal *Gesneria* species from South America is unlikely to change the reconstruction of the ancestral pollination system, because all three species have ornithophilous flowers.

Associated with pollinator transitions are changes in corolla shape that indicate a general trend for transitions from tubular to bell-shaped corollas accessible to a wide variety of visitors, including bats, moths and a few diurnal insects. In this section we focus primarily on the relationship between floral traits and bats because the pollinator importance of other visitors was not correlated with floral traits in a multivariate study of floral phenotypes (Martén-Rodríguez et al., in press). The acquisition of bat pollination is achieved through changes in corolla morphology (tubular to campanulate or subcampanulate; Fig. 6), timing of anther dehiscence (diurnal to nocturnal, Fig.7), and high 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) in hummingbird-pollinated species, 75.2 (±14.85, n=2) in bat-pollinated species and 67.1 (± 7.55, n=3) in generalists (Martén-Rodríguez and Fenster, 2008 and unpublished data). High nectar production and floral scent are considered important attractants in bat-pollinated flowers (Tschapka and Dressler, 2002); however, 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.

An unexpected floral trait that appears to evolve in conjunction with the pollination system is the presence of a tuft of trichomes at the base of stamens. The four independent origins of this trait are strictly associated with bell-shaped floral phenotypes (all subcampanulate- flowered species and one species with campanulate corolla; Fig. 9). It is possible that the stamen pubescence may play a role in keeping nectar protected from thieves in more accessible floral phenotypes. Alternatively, the hairs, which form a natural “plug” at the top of the nectar chamber, might help control nectar overflow in relatively small Gesnerieae flowers that produce copious nectar. The adaptive significance of this trait deserves further attention in future pollination studies of the Gesneriaceae.

Loss of bat pollination occurs in one lineage of generalists including *R. grandiflorum* and *R. vernicosum*, both species from 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 truly mixed phenotype with diurnal and nocturnal schedules (i.e. plants and often flowers within plants vary in timing of nectar production and anther dehiscence). *Rhytidophyllum vernicosum* also has high hummingbird visitation and importance (the product of frequency of visitation and contact with the reproductive organs of the flower), although moths appear to still contribute to pollination (Martén-Rodríguez et al., in press). Thus, this species might be in a transitional stage reverting to hummingbird pollination. No other reversals to hummingbird pollination systems are apparent in the Gesnerieae. These results parallel the findings of Perrett et al. (2003), who documented only unidirectional transitions from ornithophilous to chiropterophilous floral phenotypes in the tribe Sinningieae (Gesneriaceae), while bidirectional transitions between ornithophilous and melitophilous flowers were frequent in this South American tribe. Tripp and Manos (2008) documented a similar pattern for *Ruellia* (Acanthaceae). The change between floral phenotypes frequently involves loss of red pigmentation in the flowers of species with nocturnal pollination (bat or moth). Loss of red and purple color in flowers has been associated with "loss of function" mutations in the pathway of anthocyanin production (e.g. Mol et al., 1998); therefore, losses of color are expected to be more common than gains (Whittall et al., 2006, Rausher, 2008). Thus, while physiological constraints in flower color evolution might prevent certain pollination system transitions in some plant groups, this does not appear to be the case for the tribe Gesnerieae. The majority of species with nocturnal pollination have not lost the ability to produce floral pigments (as indicated by the presence of the red or purple markings in flowers of most bat-pollinated and generalist species; Fig. 8).

Furthermore, hummingbirds visit Gesnerieae flowers regardless of flower color, which makes other traits such as nectar production more important for pollinator attraction.

One of the most significant evolutionary trends observed in this study was the repeated origin of generalization from specialized hummingbird pollination. This transition required maintaining hummingbird visits while gaining nocturnal pollinators. Uniquely associated with this change in pollination system is the evolution of subcampanulate corollas (bell-shaped flowers with a constriction above the nectar chamber), which suggests that corolla constriction may play an important role in the maintenance of hummingbird pollination. Constricted corollas may improve pollen transfer efficiency by hummingbirds or have a yet unidentified function (Martén-Rodríguez et al., in press). Other floral traits in Gesnerieae generalists reflect adaptation to bats (bell-shaped flowers, nocturnal anther dehiscence and high nectar production). In the few systems where flowers with mixed floral characteristics are pollinated by hummingbirds and bats, it is not clear whether traits represent intermediate phenotypes or adaptations to both functional groups of pollinators (e.g. *Abutilon,* Buzato et al., 1994; *Syphocampylus sulfureus,* Sazima et al., 1994). In Gesnerieae the independent evolution of constricted corollas also occurs in conjunction with diversification in some clades. Only one transition from generalization to specialized bat pollination was observed (in *G. quisqueyana*) and this involves the evolution of a unique floral phenology where corollas are closed during the day. Therefore, the data strongly suggest that generalized pollination systems are not intermediate steps in the evolution of other pollination systems, but represent evolutionarily stable reproductive strategies.

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 and Martén-Rodríguez, 2007). In Gesnerieae autonomous self-pollination has arisen three times independently, in association with hummingbird-pollination (Fig. 9). Ecological studies suggest that this association was promoted by the low and unpredictable pollinator service by hummingbirds in the islands. Three findings support this assertion: first, hummingbird-pollinated species have the lowest frequencies of pollinator visitation (mean number of visits per flower/ per day =1 ± 1.5 SE, n=9;) when compared to bat-pollinated (mean =2 ± 1.8, n=5) and generalist species (mean =13 ± 1.8, n=5) (Martén-Rodríguez et al., in press); second, significant levels of pollen limitation (higher fruit set of hand pollinated vs. open pollinated plants) were detected only in specialized species (Martén-Rodríguez and Fenster- chapter 3); third, autonomous self-pollination provides reproductive assurance in three out of four studied hummingbird-pollinated Gesnerieae (Martén-Rodríguez and Fenster- chapter 3). *In toto*, these findings suggest that inadequate pollinator service has triggered 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-specialist Gesnerieae, which are also pollen limited, have not evolved reproductive assurance mechanisms.

Could the pattern of evolution of autonomous breeding systems in Gesnerieae be due to 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, while in protandry, pollen may be all removed by the time stigmas become receptive (Bertin, 1983; Mallick, 2001). However, an association between protogyny and autonomous selfing is not supported by empirical data; both protogyny and protandry are common among autonomous selfers, and protandry is actually more common than protogyny (reviewed by Fenster and Martén-Rodríguez, 2007). An examination of this issue in Gesnerieae shows that although autonomous selfing evolves only in protogynous lineages, the evolution of dichogamy is not associated with the evolution of particular pollination systems. For instance, protandry evolved twice from protogyny: once in a clade that has hummingbird and bat-pollinated species (*G. ventricosa* clade) and once in a hummingbird-pollinated species (*Pheidonocarpa corymbosa*). Furthermore, bat-pollinated species exhibit both protandry and protogyny. The unidirectional association between autonomous selfing and protogyny is possibly due to the fact that protogynous flowers are ancestral and the prevailing breeding system in the tribe. Therefore, there is no evidence to support the idea that differences in dichogamy states between bat and hummingbird flowers explain evolutionary trends of reproductive assurance.

An alternative explanation for the evolution of autonomous selfing in hummingbird-pollinated lineages 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. A similar trend is observed in 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 pre-adaptation to the evolution of autonomous self-pollination as a reproductive assurance mechanism. The lack of reproductive assurance mechanisms in bat-pollinated species is intriguing, particularly when considering that a recent survey found only one out of 80 species capable of autonomous selfing was bat-pollinated (Fenster and Martén-Rodríguez, 2007).

A unified view of pollination and breeding system evolution: what is special about islands?-Evolutionary transitions between functionally specialized pollination systems, where flowers evolve adaptations that restrict visitation and/ or effective pollination to specific groups of floral visitors, appear to have triggered much of the floral diversification observed in the Angiosperms (Fenster et al., 2004). However, on oceanic islands, pollinator- depauperate faunas may favor the evolution of functional generalization instead. Low diversity of floral visitors could translate into functional groups that contain one or a few species. For example, in the Dominican Republic, hummingbirds are represented by only three species, one of which is so small it cannot access nectar from typical ornithophilous flowers. This leaves the hummingbird functional group with one or two species depending on the geographic location, e.g., the Hispaniolan Emerald is the only hummingbird present at high elevations. Additionally and perhaps related to low species diversity, visitation frequencies to flowers are often lower than expected on islands. For example, while in the Costa Rican and Brazilian mainland the visitation rates to various ornithophilous Gesneriaceae ranged between three to six visits per flower per day (Martén-Rodríguez et al., in press; San Martin-Gajardo and Sazima, 2005), in Antillean Gesnerieae, hummingbird visitation is on average one visit per flower per day (Martén-Rodríguez et al., in press). Higher visitation and pollen dispersal in pollinator-rich mainland environments was also observed for *Mandevilla hirsutai* (Apocynaceae) in a comparative study of Trinidad and Tobago (Linhart and Feinsinger, 1980). Thus, natural selection should favor alternative reproductive strategies to reduce the risk of pollination failure in highly specialized species on island ecosystems. This study provides evidence for the evolution of generalization in response to low hummingbird species diversity and low frequency of visitation. However, if hummingbird pollination is not reliable in the islands, why do we observe diversification of hummingbird-pollinated lineages? i.e. how do we explain that ~ 60% of Gesnerieae species have ornithophilous flowers?

One possibility is that the evolution of autonomous self-pollination providing reproductive assurance may have allowed the maintenance and diversification of ornithophilous lineages in unpredictable pollinator environments. The occurrence of autonomous breeding systems in plants is by no means restricted to islands. However, autonomous selfing can be particularly advantageous in such ecosystems after long-distance dispersal, or anytime when pollinators and/ or mates are scarce (Baker, 1955; Jain, 1979). In Gesnerieae, low pollinator abundance rather than mate scarcity appears to be the selective pressure favoring the evolution of autonomous selfing. While two of the most frequent evolutionary transitions in flowering plants involve the evolution of pollination systems (Fenster et al., 2004; Wilson et al., 2006) and the evolution of selfing from outcrossing (Stebbins, 1974; Fenster and Barrett, 1994), studies of pollination and breeding system evolution have traditionally run along separate pathways (Fenster and Martén-Rodríguez, 2007). This study demonstrates that the simultaneous study of both pollination and breeding systems can provide illumination to our understanding of the evolution of floral diversity.

While this work sheds light on a number of factors that could explain the diversification of Antillean Gesneriaceae, it raises many more questions: for instance, bat-pollinated specialists from Puerto Rico and Hispaniola were pollen limited and had no reproductive assurance; however, significant diversification of bat-pollinated species occurs in one lineage from Jamaica. Does this indicate that reproductive assurance mechanisms might be present in the Jamaican chiropterophilous species? Or is bat pollination more efficient in Jamaica due to the occurrence of large underground cave systems hosting colonies of nectar-feeding bats? With regard to generalist species from high elevations, is evolutionary specialization towards hummingbird pollination occurring due to the absence of nectar-feeding bats in these environments? Furthermore, what is the functional significance of subcampanulate corollas? Do they promote effective pollination by hummingbirds while allowing for bat pollination? Do the tubular and funnel-shaped white flowers of Cuban *G. humilis* and Jamaican *G. pumila* represent independent origins of bee pollination*?* This study underscores the usefulness of combining phylogeny with field-based ecological studies to provide further insights onto the environmental factors that can influence floral evolution.

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TABLE 1. Summary statistics from parsimony analyses for five data sets, and models of nucleotide substitution used in ML analyses of the tribe Gesnerieae.

Data set	N^o species	N^o characters	N^o parsimony informative	N^o trees	Tree length	CI	RI
ITS	37	632	96	349	293	0.78	0.81
GCYC	35	514	26	98	103	0.94	0.93
ITS + GCYC	35	1146	116	48	416	0.78	0.77
Morphology	37	37	37	32	178	0.30	0.65
Morphology + ITS + GCYC	35	1187	160	50	624		

FIGURE 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 leucomallon*, subcampanulate diurnal/nocturnal flowers – pollinated by bats, hummingbirds and moths (D) *Bellonia aspera* – bee-pollinated.

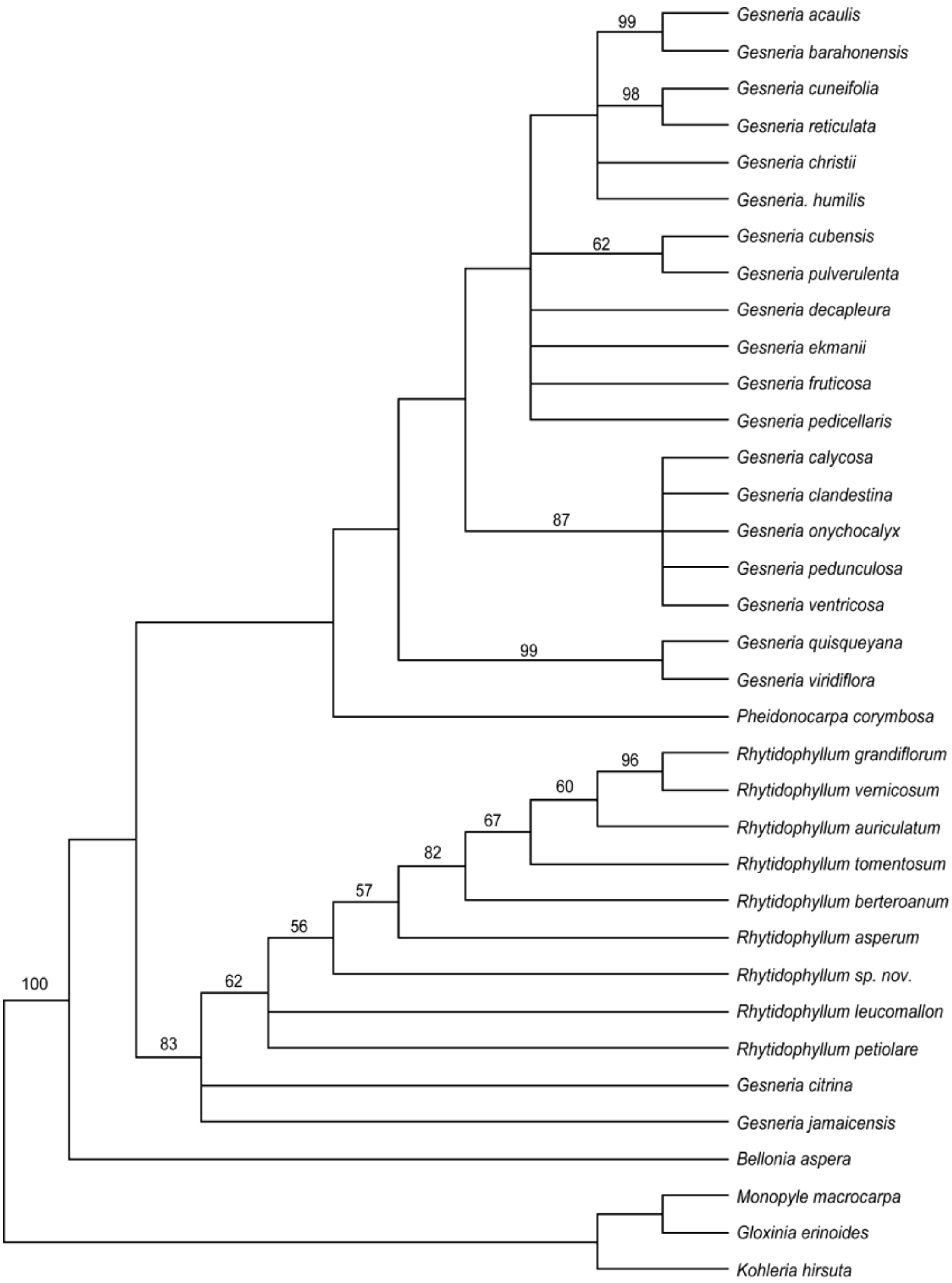


FIGURE 2. Strict consensus of 1905 most parsimonious trees (Length= 416) from analysis of the combined ITS+ *GCYC* dataset. Numbers indicate branch support where bootstrap values ≥ 50%.

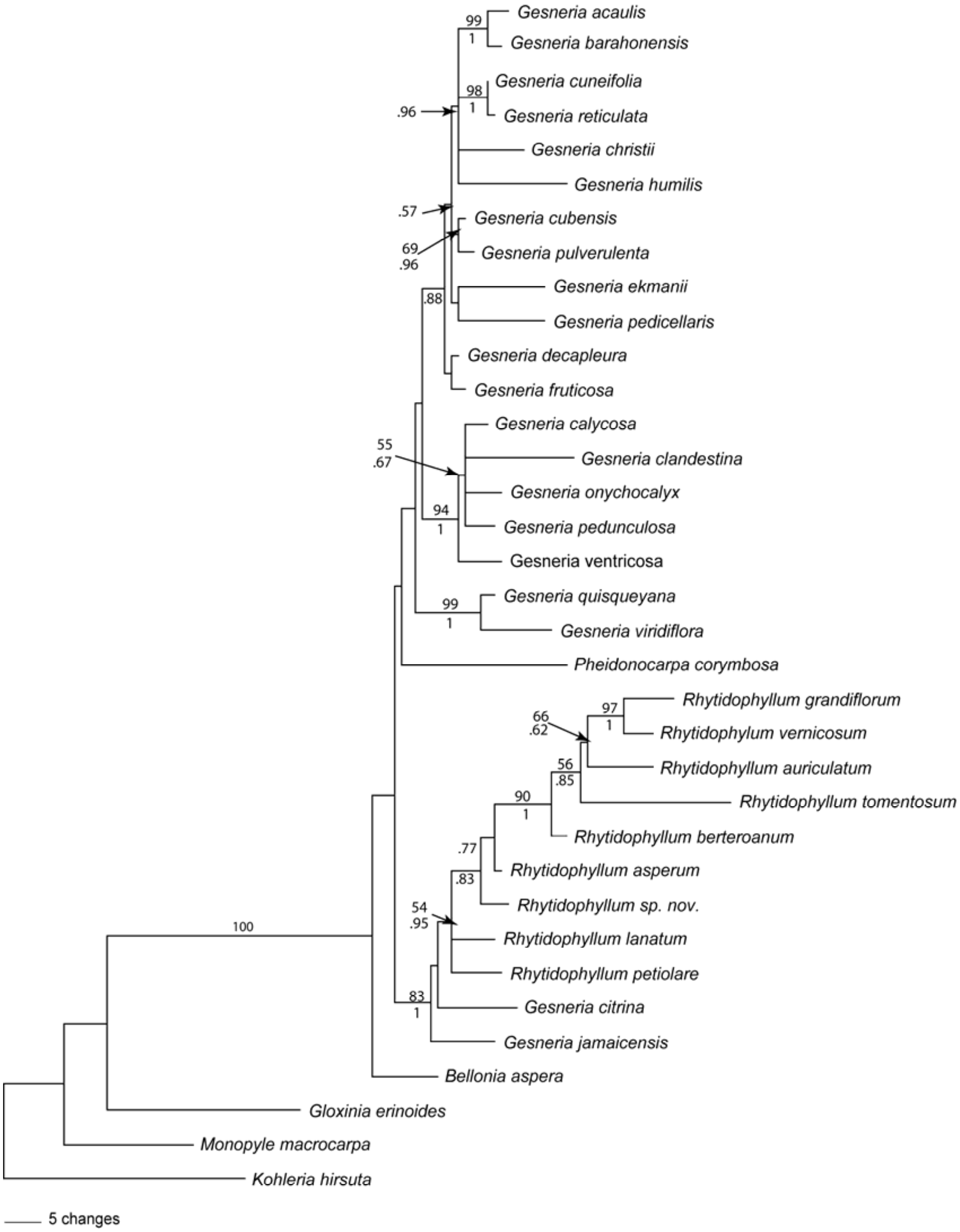


FIGURE 3. One of the three Maximum Likelihood consensus trees of equal likelihood (-ln L= 4197.06944) obtained from analysis of combined ITS and *GCYC* sequences of the Gesnerieae. Numbers indicate branch support where bootstrap values ≥ 50% and posterior probabilities from MR Bayes ≥ 0.5.

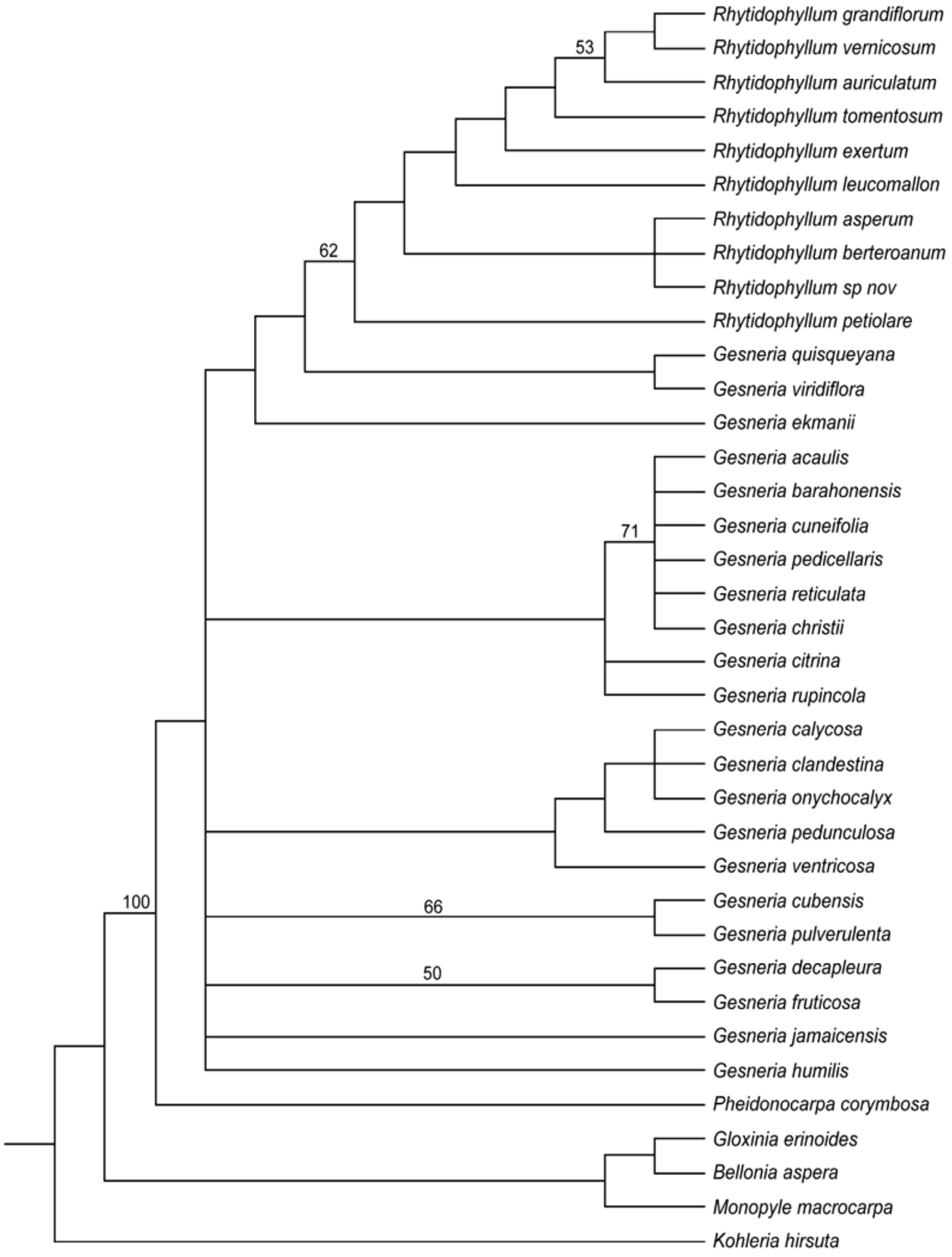


FIGURE 4. Strict MP consensus of 32 most parsimonious trees of 178 steps from analysis of Gesnerieae morphology dataset. Numbers above branches indicate branch support where bootstrap values are ≥ 50%.

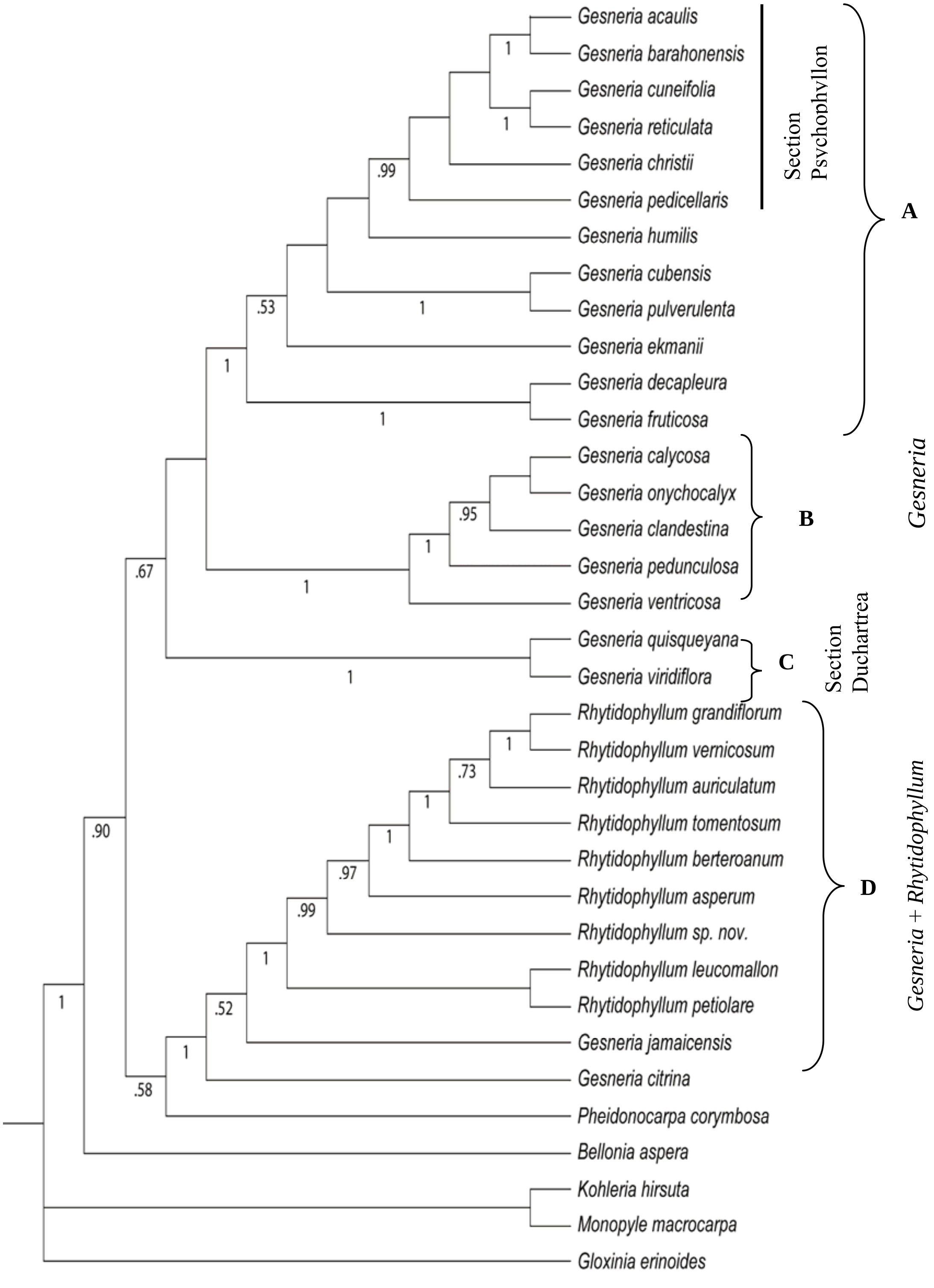


FIGURE 5. Bayesian analysis of the total evidence data set for the tribe Gesnerieae. Numbers above branches indicate branch support where posterior probabilities are above 50%.

Appendix I. Morphological characters and character states used in phylogenetic analyses of the tribe Gesnerieae.

1.Chromosome number: (0) *n*=13, (1) *n* =14, (2) n =28; **2. Growth habit:** (0) herbs, (1) subshrubs (woody only at base), (2) woody subshrubs or shrubs; **3. Posture:** (0) erect, (1) often decumbent or pendant; **4. Rhizomes:** (0) absent, (1) present; **5. Lenticels:** (1) absent, (2) present; **6. Leaf phyllotaxy:** (0) opposite or ternate, (1) alternate/spiral; **7. Leaf clustering:** (0) not clustered, (1) distinctly clustered at the end of branches; **8. Apical mersitem:** (0) normal, (1) claw-like; **9. Pseudostipules:** (1) absent, (2) present; **10. Petiole vasculature:** (0) shallow crescent, (1) a ring or almost complete ring; **11. Leaf shape:** (0) broadest below the middle, (1) broadest at the middle, (2) broadest above the middle; **12. Leaf veins, number:** (0) 3–5 pairs, (1) 6–8 (9) pairs, (2) 10–20 pairs; **13. Leaf margins:** (0) serrate-dentate, (1) aculeate, (3) serrulate/ crenulate; **14. Leaf texture:** (0) soft/ membranous, (1) stiff/ coriaceous; **15. Adaxial surface texture of leaf:** (0) plane, (1) bullate, (2) bullulate; **16. Stomatal domes:** (0) absent, (1) present; **17. Stomatal islands:** (0) not aggregated (i.e., randomly scattered), (1) aggregated; **18. Cell wall sinuation:** (0) straight; **19. Abaxial indumentum:** (0) absent, (1) pilose, (2) hirsute/hispid, (3) lanate; **20. Peduncle:** (0) short (usually between 2-30 mm), (1) long (usually between 60-200 mm); **21. Peduncle shape:** (0) terete, (1) sulcate, (2) flattened; **22. Flower number:** (0) solitary, (1) one to four flowers, (1) many; **23. Calyx lobe shape:** (0) linear/lanceolate, (1) triangular, (2) ovate; **24. Corolla shape**: (0) tubular, (1) campanulate, (2) subcampanulate, (3) rotate, (4) funnelform; **25.Corolla carotenoids:** (0) absent, (1) present; **26.Corolla anthocyanines:** (0) absent, (1) present; **27.Distribution of anthocyanines:** (0): even (i.e. corolla entirely red or pink), (1) spotted (i.e. red or purple markings on corolla limb); **28.Corolla symmetry:** (0) zygomorphic, (1) subactinomorphic, (2) actinomorphic; **29. Corolla indumentum external:** (0) absent, (1) present; **30. Corolla indumentum internal:** absent, (1) present; **31. Stamen insertion:** (0) stamens inserted at the base of the corolla (i.e. filaments not adnate to corolla > 1mm), (1) stamens distinctly adnate to base of the corolla (> 2mm); **32. Stamen length:** (0) stamens not exerted beyond corolla mouth, (1) stamens distinctly exerted beyond corolla mouth; **33. Filament**

pubescence: (0) filaments glabrous, (1) filaments sparsely pubescence, (2) filaments densely pubescent at base; **34. Stigma shape:** (0) stomatomorphic, (1) bilobed, (2) capitate, (3) clavate; **35. Ovary position:** (0) inferior, (1) half inferior; **36. Capsule external texture:** (0) costae obscure or absent, (1) costae prominent; **37. Dichogamy state:** (0) adichogamous, (1) protogynous. The following traits were used in character reconstruction only: **38. Pollination system:** (0) Bats, (1) Hummingbirds, (2) Moths, (3) Bees, (4) Generalist insects; **39. Timing of anther dehiscence and nectar production**: (0): diurnal, (1) nocturnal; **40. Flower color:** (0) White, (1) Green, (2) yellow, (3) pink, (4) red, (5) Dark red marks; **41. Reproductive assurance:** index of autonomous self-pollination-IA (1- [fruit set bagged flowers/ fruit set outcrossed flowers]), (0) Potential RA< 10%, (1) Potential RA> 10%., **42. Geographic distribution:** (0) Cuba, (1) Jamaica, (2) Hispaniola, (3) Puerto Rico, (4) Lesser Antilles, (5) South America.

Appendix II. Morphological data matrix of 34 Gesnerieae species and three Gloxinieae outgroups. Characters and character states are specified in appendix I.

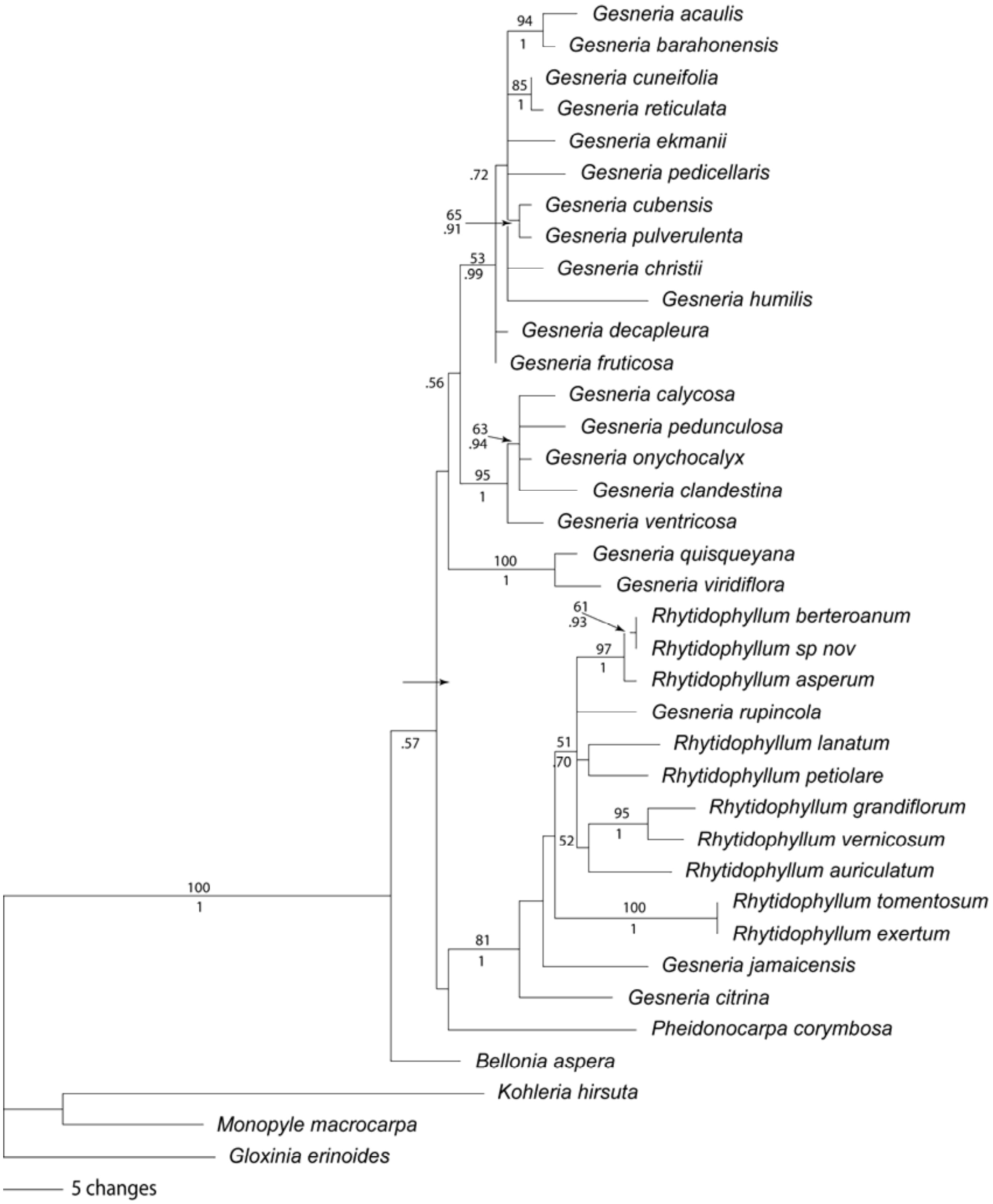
Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Species																									
Kohleria hirsuta	0	0	1	1	0	0	0	0	0	0	2	1	1	0	0	1	0	0	2	0	0	1	0	0	1
Monopyle macrocarpa	0	0	0	1	0	0	0	0	0	0	2	2	0	0	0	0	0	1	0	0	0	0	0	1	0
Gloxinia erinoides	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	2	1	0	2	0	0	0
Bellonia aspera	0	2	0	0	1	0	0	0	0	0	0	0	0	1	0	?	?	1	2	0	0	1	0	3	0
Gesneria acaulis	1	1	1	0	0	1	1	0	0	1	1	1	0	0	1	1	1	0	1	0	0	2	1	0	1
Gesneria barahonensis	?	1	1	0	0	1	1	0	0	1	1	1	0	0	1	?	?	?	1	0	0	2	1	0	1
Gesneria calycosa	?	2	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	2	0	0	1	0
Gesneria citrina	1	2	1	0	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	0	0	2	0	0	1
Gesneria clandestina	?	2	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Gesneria cubensis	?	2	0	0	1	1	1	0	0	1	1	1	0	1	0	?	1	?	0	0	0	1	0	0	0
Gesneria cuneifolia	2	1	1	0	0	1	1	0	0	1	1	1	0	0	1	1	1	0	1	0	0	1	1	0	1
Gesneria decapleura	?	2	0	0	0	1	0	0	0	1	1	1	1	0	0	?	?	?	1	1	0	1	1	0	1
Gesneria ekmanii	?	2	0	0	0	1	1	0	0	1	0	2	0	1	0	?	1	0	0	1	0	1	1	2	0
Gesneria fruticosa	?	2	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	0	1	1	0
Gesneria jamaicensis	1	2	1	0	0	1	0	0	0	1	0	2	0	1	0	0	1	0	0	1	0	0	0	0	0
Gesneria onychocalyx	?	2	0	0	1	1	0	0	0	1	0	1	0	0	0	?	?	?	0	0	0	0	0	1	0
Gesneria pedicellaris	?	1	1	0	0	1	1	0	0	1	1	2	0	0	1	1	1	0	0	0	0	2	0	0	1
Gesneria pedunculosa	1	2	0	0	1	1	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	1	0	1	0
Gesneria pulverulenta	?	2	0	0	1	1	1	0	0	1	1	1	0	1	0	?	?	?	0	0	0	1	0	0	0
Gesneria pumila	1	1	1	0	0	1	1	0	0	1	1	1	0	0	0	1	1	0	0	0	0	1	0	4	0
Gesneria quisqueyana	?	2	0	0	1	1	1	1	0	1	0	2	2	1	0	?	?	?	0	1	0	2	2	2	0
Gesneria reticulata	1	1	1	0	0	1	1	0	0	1	1	1	0	0	1	?	?	?	0	0	0	1	2	0	1
Gesneria ventricosa	1	2	0	0	1	1	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	1	0	0	1
Gesneria viridiflora	?	2	0	0	1	1	1	1	0	1	0	2	2	1	0	1	1	0	0	1	0	2	2	2	0
Pheidonocarpa corymbosa	1	2	1	0	0	0	0	0	0	1	2	1	0	1	0	0	1	0	1	1	0	2	1	0	1
Rhytidophyllum asperum	?	2	0	0	0	1	1	0	0	1	0	2	2	1	2	1	0	1	2	0	0	1	0	0	1
Rhytidophyllum auriculatum	1	2	0	0	0	1	0	0	1	1	0	2	2	0	2	1	0	1	2	1	1	2	1	2	1
Rhytidophyllum berteroanum	1	2	0	0	1	1	1	0	0	1	0	2	2	0	2	1	0	1	2	1	0	2	0	0	1
Rhytidophyllum sp. nov.	?	2	0	0	1	1	1	0	0	1	0	2	2	0	2	?	?	?	2	1	0	2	0	2	1
Rhytidophyllum grandiflorum	?	2	0	0	1	1	0	0	1	1	0	2	2	1	2	1	0	1	2	1	1	2	1	2	1
Rhytidophyllum leucomallon	1	2	0	0	0	1	0	0	0	1	0	2	2	0	2	1	0	1	3	1	0	2	2	2	1
Rhytidophyllum petiolare	?	2	1	0	0	1	1	0	0	1	0	2	2	0	0	?	?	?	2	1	0	2	2	2	0
Rhytidophyllum tomentosum	1	2	0	0	0	1	0	0	1	1	0	2	2	0	2	1	0	1	3	1	0	2	1	2	1
Rhytidophyllum vernicosum	?	2	0	0	0	1	0	0	0	1	0	2	2	1	2	1	0	1	2	1	1	1	1	2	1
Gesneria christii	1	1	1	0	0	1	1	0	0	1	1	1	0	0	1	1	1	0	0	0	0	1	0	0	1
Gesneria humilis	?	2	0	0	0	1	1	0	0	1	1	1	0	0	0	0	1	0	1	0	0	1	1	0	0
Gesneria rupincola	?	2	1	0	0	1	0	0	0	1	1	1	0	0	2	1	1	0	2	0	0	2	0	0	1
Rhytidophyllum exertum	?	2	1	0	0	1	0	0	0	1	0	2	2	0	2	1	0	1	2	1	0	2	1	2	1

Character	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Species																	
Kohleria hirsuta	1	0	0	0	1	0	0	0	1	1	0	2	0	0	0	?	5
Monopyle macrocarpa	0	1	0	0	0	0	0	0	2	1	1	0	3	0	4	?	5
Gloxinia erinoides	1	1	0	0	1	0	0	0	2	1	1	?	4	0	0/4	?	5
Bellonia aspera	0	-	2	0	1	0	1	0	2	1	1	0	3	0	4	0	0/2
Gesneria acaulis	1	0	0	0	1	0	0	0	3	0	1	1	0	0	0	?	1
Gesneria barahonensis	1	0	1	0	1	0	0	0	3	0	1	1	0	0	0	?	2
Gesneria calycosa	0	-	0	0	0	0	1	0	0	0	1	2	1	1	4	?	1
Gesneria citrina	0	-	0	0	1	0	1	0	3	0	1	1	0	0	2	1	3
Gesneria clandestina	0	-	0	0	1	0	0	0	0	0	1	2	1	?	4	?	1
Gesneria cubensis	1	0	1	0	0	0	1	0	?	0	1	?	0	0	0	?	0/2
Gesneria cuneifolia	1	0	1	0	1	0	0	0	3	0	1	1	0	0	0	1	3
Gesneria decapleura	1	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	2
Gesneria ekmanii	1	1	0	0	0	0	0	0	0	0	1	1	0/2	1	4	0	2
Gesneria fruticosa	2	1	0	0	1	0	0	2	0	0	1	1	1	1	3/5	0	2
Gesneria jamaicensis	1	0	0	0	0	0	0	0	1	0	0	?	0	0	1	?	1
Gesneria onychocalyx	0	-	0	0	0	0	1	0	0	0	1	?	1	1	3	?	1
Gesneria pedicellaris	1	0	1	0	1	0	0	0	3	0	1	1	0	0	0	1	2
Gesneria pedunculosa	0	-	0	0	1	0	1	0	0	0	1	2	1	1	4	0	3
Gesneria pulverulenta	1	0	1	0	0	0	0	0	2	0	1	?	0	0	0	1	2
Gesneria pumila	1	-	0	0	1	0	0	0	3	0	0	?	?	1	4	?	1
Gesneria quisqueyana	1	1	0	1	0	0	0	2	0	0	0	1	1	1	3/5	0	2
Gesneria reticulata	1	0	1	0	1	0	0	0	3	0	1	1	0	0	0	1	0/2/3
Gesneria ventricosa	1	0	0	0	0	0	1	1	0	0	1	2	0	0	0	?	4
Gesneria viridiflora	1	1	0	1	0	0	0	2	0	0	0	1	0/1/2	1	3	0	0/3
Pheidonocarpa corymbosa	1	0	0	0	1	0	0	1	1	1	0	2	0	0	0	?	0/1
Rhytidophyllum asperum	1	0	0	0	1	1	0	0	2	0	0	1	0	0	0	1	2
Rhytidophyllum auriculatum	1	1	0	0	1	1	0	2	2	0	0	1	0/1/2	1	2/5	0	2/3
Rhytidophyllum berteroanum	1	0	0	0	1	1	0	0	3	0	0	1	?	0	0	?	2
Rhytidophyllum sp. nov.	1	0	0	0	1	1	0	0	2	0	0	1	0	?	0	?	2
Rhytidophyllum grandiflorum	1	1	0	0	1	1	0	2	2	0	0	1	0/2	1	2/5	?	2
Rhytidophyllum leucomallon	1	-	0	0	0	1	0	2	2	0	0	1	0/1/2	1	2	0	2
Rhytidophyllum petiolare	0	-	0	0	1	1	0	2	2	0	0	1	?	?	4	?	2
Rhytidophyllum tomentosum	1	1	0	0	1	1	0	2	2	0	0	1	?	1	2/5	?	1
Rhytidophyllum vernicosum	1	1	0	0	1	1	0	2	2	0	0	1	0/2	0/1	2/5	0	2
Gesneria christii	1	0	1	0	1	0	0	0	1	0	1	1	?	1	0	?	2
Gesneria humilis	0	-	0	0	0	0	0	0	0	0	1	?	?	?	4	?	0/2
Gesneria rupincola	1	0	0	0	0	0	0	1	?	0	?	?	?	0	0	?	0
Rhytidophyllum exertum	1	1	0	0	1	1	1	1	2	0	0	1	?	1	2/5	?	0

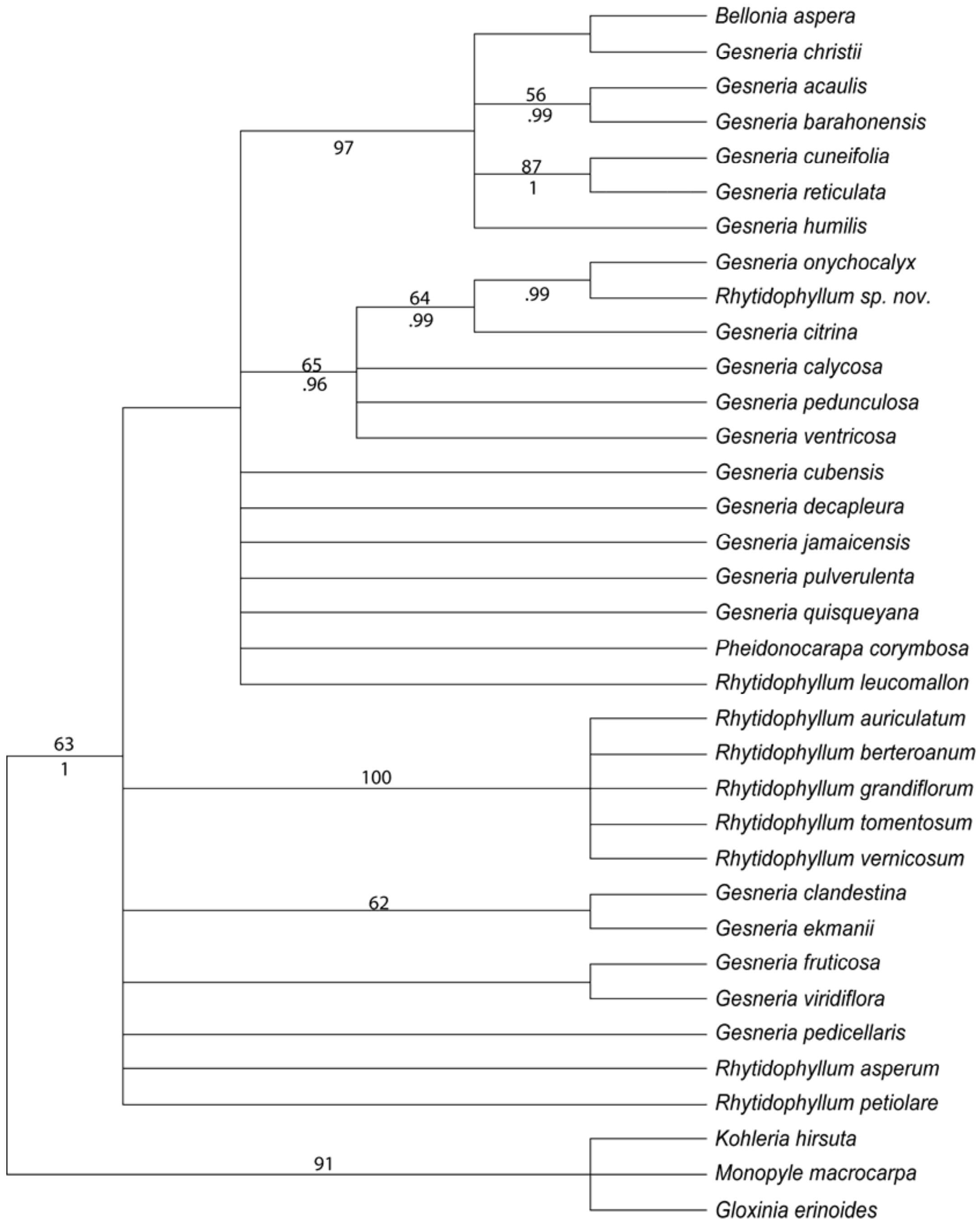
Appendix III. List of species, collection numbers and Genbank accession numbers used in phylogenetic analyses of the tribe Gesnerieae.

Species	Collector	Vocher #	ITS	*GCYC*
Bellonia aspera Linneaus	SMR	1233	√	√
Gesneria acaulis Linneaus	SMR	1188	√	√
Gesneria barahonensis Urban	SMR	1239	√	√
Gesneria calycosa (Hooker) O.Kuntze	SMR	1190	√	√
Gesneria clandestina (Grisebach) Urban	SMR	1197	√	√
Gesneria citrina Urban	SMR	1246	√	√
Gesneria cubensis (Decaisne) Baillon	SMR	1232	√	√
Gesneria cuneifolia (A.P. de Candolle) Fritsch	SMR	1247	√	√
Gesneria decapleura Urban	SMR	1240	√	√
Gesneria ekmanii Swartz	P.Acevedo	13892	√	√
Gesneria fruticosa (Linneaus) O.Kuntze	SMR	1227	√	√
Gesneria jamaicensis N.Britton	SMR	1193	√	√
Gesneria onychocalyx L.E.Skog	SMR	1195	√	√
Gesneria pedicellaris Alain	SMR	1229	√	√
Gesneria pedunculosa (A.P.de C) Fritsch	SMR	1198	√	√
Gesneria pulverulenta Alain	SMR	1237	√	√
Gesneria quisqueyana Alain	SMR	1230	√	√
Gesneria reticulata (Grisebach) Urban	SMR	1248	√	√
Gesneria ventricosa Swartz	SMR	1112A	√	√
Gesneria viridiflora (Decaisne) O.Kuntze	SMR	1199	√	√
Pheidonocarpa corymbosa (Swartz) L.E.Skog	SMR	1192	√	√
Rhytidophylum auriculatum H.Hooker	SMR	1200	√	√
Rhytidophyllum asperum Alain	SMR	1235	√	√
Rhytidophyllum berteroanum Martius	SMR	1226	√	√
Rhytidophyllum sp. nov.	F. Jimenez		√	√
Rhytidophyllum grandiflorum ined.	SMR	1224	√	√
Rhytidophyllum leucomallon Hanstein	P.Acevedo	13966	√	√
Rhytidophyllum petiolare A.P. de Candolle	SMR	1228	√	√
Rythidophyllum tomentosum (Linneaus) Martius	SMR	1191	√	√
Rhytidophyllum vernicosum Urban & Eckman	P.Acevedo	13963	√	√
Gesneria christii Urban			AY047046	AY363923
Gesneria humilis Linneaus			AY047051	AY423156
Gesneria rupincola Urban			AY047057	
Rhytidopyllum exertum Grisebach			AY047055	
Kohleria hirsuta			AY702374	AY623138
Monopyle macrocarpa			AY047070	AY623148
Gloxinia erinoides			AY047073	AY623149

Appendix IV. Maximum Likelihood tree (-ln L=2590.58461) obtained from analysis of the internal transcribed space (ITS) dataset including 34 Gesnerieae species and 3 Gloxinieae outgroups. Numbers indicate branch support where bootstrap values ≥ 50% and posterior probabilities ≥ 0.5.



Appendix V. Maximum Likelihood consensus tree of five trees (-ln L=2590.58461) obtained from analysis of *GCYC* sequences of the tribe Gesnerieae. Numbers indicate branch support where bootstrap values ≥ 50% and posterior probabilities from Mr Bayes ≥ 0.5.



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