doi:10.1111/j.1558-5646.2008.00398.x

IS FLORAL SPECIALIZATION AN EVOLUTIONARY DEAD-END? POLLINATION SYSTEM TRANSITIONS IN *RUELLIA* (ACANTHACEAE)

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Received November 7, 2007
Accepted March 17, 2008

> ***"...the highly developed, or specialized types of one geologic period have not been the parents of the types of succeeding periods, but ...the descent has been derived from the less specialized of preceding ages..."***
> **— Edward D. Cope (1896), pp. 173–174**

Pollination systems frequently reflect adaptations to particular groups of pollinators. Such systems are indicative of evolutionary specialization and have been important in angiosperm diversification. We studied the evolution of pollination systems in the large genus *Ruellia*. Phylogenetic analyses, morphological ordinations, ancestral state reconstructions, and a character mapping simulation were conducted to reveal key patterns in the direction and lability of floral characters associated with pollination. We found significant floral morphological differences among species that were generally associated with different groups of floral visitors. Floral evolution has been highly labile and also directional. Some specialized systems such as hawkmoth or bat pollination are likely evolutionary dead-ends. In contrast, specialized pollination by hummingbirds is clearly not a dead-end. We found evidence for multiple reverse transitions from presumed ancestral hummingbird pollination to more derived bee or insect pollination. These repeated origins of insect pollination from hummingbird-pollinated ancestors have not evolved without historical baggage. Flowers of insect-pollinated species derived from hummingbird-pollinated ancestors are morphologically more similar to hummingbird flowers than they are to other more distantly related insect-pollinated flowers. Finally, some pollinator switches were concomitant with changes in floral morphology that are associated with those pollinators. These observations are consistent with the hypothesis that some transitions have been adaptive in the evolution of *Ruellia*.

KEY WORDS: **Dollo's law, flower, generalist, pollinator, specialist, syndrome.**

Specialization in pollination systems has been a central factor in the diversification of angiosperms and their flowers (Grant and Grant 1965; Faegri and van der Pijl 1966; Stebbins 1970; Eriksson and Bremer 1992, but see Waser et al. 1996). For example, specialization onto different pollinators has been found to be a primary mechanism for reproductive isolation between sympatric species pairs (Hodges and Arnold 1994; Kay and Schemske 2003; Ramsey et al. 2003; Kephart and Theiss 2004; Gegear and Burns 2007). Lineages that are animal pollinated have also been linked to higher species diversity (Erikkson and Bremer 1992; Dodd et al. 1999). Floral specialization is intriguing because changes in ecological niche such as adaptations to new pollinators are potentially linked to reproductive isolation and thus may directly affect speciation (Schemske and Bradshaw 1999).

A long-standing hypothesis in evolution is that ecological generalists give rise to specialists more often than the reverse transition (Futuyma and Moreno 1988). This hypothesis derives from the idea that because organisms are constantly facing unpredictable and unfavorable environments, there must exist some measure of selection on phenotypes that are less limited by resource availability or have broader ecological niche widths (Futuyma and Moreno 1988; Kassen 2002). Generalists also have a greater potential to colonize new habitats and give rise to new lineages, and may be less prone to extinction (Zayed et al. 2005; Miller et al. 2006; Hoehn et al. 2007). Further, a lineage that has evolved numerous adaptations to a narrow resource becomes increasingly committed to that state, making reversals to generalized habits or shifts to different specialized states very unlikely (Futuyma and Moreno 1988). Specialization is generally thought to arise because "the jack of all trades," that is, the generalist, "is the master of none" (Levins 1968; MacArthur 1972; for examples, see Levins 1962; Bolnick 2004; MacLean et al. 2005; Muchhala 2007; Ostrowski et al. 2007). If specialists always and only represent derived states, then there is validity to the claim that specialization is an evolutionary dead-end (Cope 1896; Mayr 1963; for examples, see Buckling et al. 2003: bacteria; Morse and Farrell (2005): parasitoid beetles; Nosil 2002 in part: phytophagous insects; Stephens and Wiens 2003: turtles; Toft 1995: poison-dart frogs). Other studies, however, have challenged this dogma (D'Haese 2000: springtails; Elliott et al. 1999: anemonefish; Holmes 1977: tetrapod jaw bones and bird wings; Nosil 2002 in part: phytophagous insects; Nosil 2005: phytophagous insects; Piano et al. 1997: Hawaiian *Drosophila*; Stireman 2005: parasitoid flies; Yotoko et al. 2005: parasitic flies). Because plants and their pollinators represent some of the most diverse lineages globally, floral specialization may offer the greatest opportunity to address this question.

Two concepts in floral evolution are often contemplated: the direction and lability of character transitions. Trends in polarity and lability have been established by a long history of research in pollination biology. For example, a common hypothesis is that specialized pollination systems (e.g., hummingbird pollination) are usually derived and generalized systems (e.g., bee/insect pollination) are ancestral (Grant 1993). Yet this is mostly conventional wisdom and has not been the focus of explicit study and hypothesis testing. Our understanding of trends in floral evolution is still tenuous owing to discoveries of reverse or "unorthodox" transitions (McDade 1992; Stein 1992; Goldblatt and Manning 1996; Armbruster 1998; Perret et al. 2003).

Species of *Ruellia* (ca. 300 spp.) are known for their floral diversity. Many flowers are brightly colored due to the production of purple and red anthocyanins (Bloom 1976). Primary pollinators include bees, hummingbirds, hawkmoths, bats, and also butterflies. In *Ruellia* and other plants, "bee or insect flowers" have purple corollas with large lobes that serve as landing platforms, short tubes or throats, and open floral morphologies that permit access by nectaring bees. Further and most importantly, the inserted position of male and female reproductive parts allows many other floral visitors, for example, hummingbirds and other insects, to make contact with anthers and stigmas upon visitation. In contrast, "hummingbird flowers" are characterized by species with red corollas, reflexed lobes that are held out of the way of floral visitors, generally long tubes or throats that contain nectar deep within flowers, and exserted reproductive parts that prevent most visitors other than hummingbirds (and maybe other large animals) from making contact with them. Thus, floral architecture alone dictates that species with "hummingbird flowers" are likely more specialized than species with "bee flowers." In *Ruellia*, other specialized pollination systems include hawkmoth and bat pollination. Hawkmoth flowers open at night, are white, and have very long corolla tubes. Unless nectar is robbed or removed illegitimately, only hawkmoths have tongues (probosces) long enough to access nectar. Bat flowers also open at night, are pale colored (yellow to green), have very wide corolla throats and tubes to permit access by bats, and very long-exserted reproductive parts. This ecological and morphological diversity of species of *Ruellia* creates the opportunity for our study.

To date, there have been only a small number of studies to examine changes in both floral morphology and pollinator systems within a phylogenetic context (Table 1). These studies have greatly benefited our understanding of macroevolutionary trends in the direction and lability of floral character evolution, and also provide the first opportunity in pollination biology to rigorously test the age-old "specialized states are dead-ends" hypothesis. Based on studies from Table 1 that included both hummingbird- and bee- or insect-pollinated species ($N = 11$), this hypothesis is supported: the overwhelming pattern is transitions from bee/insect toward hummingbird pollination, with only one study documenting the reverse (Perret et al. 2003). Although we do not address caveats associated with patterns in floral evolution, such patterns must in part depend upon various factors such as the strength of phylogenetic or niche conservatism, clade size, or the geographical distribution of the plant lineage.

How might these studies give a restricted view of possible transitions? Limitations include the taxonomic level at which the study was conducted, relative lineage size, and rigor of methods and tests. Although explorations of intrageneric transitions are among the most powerful approaches for studying character evolution (Barraclough et al. 1999; Endress 2003; Perret et al. 2003), fewer than half of the unambiguous 11 bee (or insect)/hummingbird studies were executed strictly at the species level. Second, studies of species-rich lineages allow for maximum application of comparative methods, yet again, fewer than half of the 11 studies focused on large genera (> ca. 100 spp.). The

Table 1. Survey of studies that examined pollinator switches within a phylogenetic context. The second column refers to the proportion of taxa sampled relative to clade size for the focal plant lineage. The third column indicates whether the observed transition(s) might be considered a reverse transition.

Focal plant lineage	Approx. no. of taxa sampled/ clade size	Unorthodox	Major conclusion(s)	Reference
Loasoideae (Loasaceae)	38/200	No	Ancestral pollination by short-tongued bees, multiple transitions (probably > 5) to pollination by hummingbirds and long-tongued bees	Ackermann and Weigend (2006)
Dalechampia (Euphorbiaceae)	39–46/120	No	Ancestral species of *Dal*echampia pollinated by pollen-collecting or fragrance-collecting euglossine bees, one transition to resin-collecting bees, three to four additional transitions to pollination by euglossine bees	Armbruster (1992, 1993, 1996a)
Dalechampia (Euphorbiaceae)	46/120	No	Some components of floral morphology evolved in response to pollinators	Armbruster (1996b)
Dalechampia (Euphorbiaceae)	42/120	No	Exaptations have been an important to the evolution of new pollination systems, for example, floral resins evolved as defense mechanism, later co-opted for reward by resin-collecting bee pollinators	Armbruster (1997)
Dalechampia (Euphorbiaceae)	24/120	Yes	One transition from pollination by specialized resin-collecting bees to generalized pollination by wide range of insects, regarded as first study to document "reverse" transition in pollination biology	Armbruster (1998)
Adansonia (Bombacaceae)	8/8	No	One transition between pollination by hawkmoths and mammals, but direction equivocal	Baum et al. (1998)
Mimulus sect. *Erythranthe*	6/6	No	Insect pollination ancestral, two transitions to hummingbird pollination	Beardsley et al. (2003)
Erythrina (Fabaceae)	39/112	?	Passerine bird pollination ancestral, four transitions to hummingbird pollination	Bruneau (1997)
Catasetinae and Cyrtopodiinae (Orchidaceae)	54/575	No	Ancestral pollination by male and female euglossine bees a preadaptation to current pollination by males only	Chase and Hills (1992)
Antirrhineae (Scrophulariaceae)	19/328	No	Ancestral pollination by bees, three transitions to hummingbird pollination	Ghebrehiwet et al. (2000)
Schoenocephalieae (Rapateaceae)	3/7	No	Ancestral pollination by bees, one transition to hummingbird pollination	Givnish et al. (2000)
Lapeirousia (Iridaceae)	21/21	Yes	Apparent reversal of perianth tube length, (long to short), corresponding to shift from pollination by long-tongued flies and sphinx moths to pollination by bees and noctuid moths	Goldblatt and Manning (1996)
Platanthera (Orchidaceae)	33/85	?	Switches from pollination by settling moths to pollination by nocturnal and diurnal hawkmoths, mosquitoes, butterflies, flies, and bees	Hapeman and Inoue (1997)
Streptocarpus and *Saintpaulia* (Gesneriaceae)	53/147	?	Ancestral pollination probably by flies, four transitions to different types of bee pollination, three transitions to moth or butterfly pollination, one transition to bird pollination	Harrison et al. (1999)

Continued.

Table 1. Continued.

Focal plant lineage	Approx. no. of taxa sampled/ clade size	Unorthodox	Major conclusion(s)	Reference
Disa (Orchidaceae)	27/120	No	19 different pollination systems (different insects lineages) across 27 species	Johnson et al. (1998)
Passiflora Murucuja group (Passifloraceae)	10/12	No	Ancestral pollination by insects, one transition to hummingbird pollination and one transition from hummingbird to bat pollination	E. Kay (unpubl. data, pers. comm.)
Costus (Costaceae)	41/51	No	Ancestral pollination by bees, at least seven transitions to hummingbird pollination	Kay et al. (2005)
Melianthus (Melianthaceae)	8/8	No	Pollinated by wide variety of birds, floral diversity probably driven by factors other than pollinators	Linder et al. (2006)
Parkia (Fabaceae)	31/31	No	Insect pollination ancestral, one transition to bat pollination	Luckow and Hopkins (1995)
Aphelandra pulcherrima complex (Acanthaceae)	30/45	Yes	Pollination by generalist trochiline derived from pollination by specialized hermit hummingbirds	McDade (1992)
Calochortus (Calochortaceae)	62/67	No	Most species are generalists, although lots of transitions between floral systems, unlikely it has been adaptive	Patterson and Givnish (2004)
Schizanthus (Solanaceae)	12/12	No	Ancestral pollination by bees, one transition each to hummingbird and hawkmoth pollination	Pérez et al. (2006)
Sinningieae (Gesneriaceae)	76/82	Yes	Ancestral pollination by hummingbirds or bees, but equivocal; multiple transitions from hummingbirds to bees, bats, and one to moths, two to three transitions from pollination by bees to birds, and one from bees to bats	Perret et al. (2003)
Centropogon subgen. *Centropogon* (Campanulaceae)	4/55	Yes	One to two transitions from pollination by specialized sicklebill hummingbird to pollination by generalist hermit hummingbird	Stein (1992)
Nasa (Loasaceae)	60/>100	No	Ancestral pollination by bees with two transitions to pollination by hummingbirds	Weigend and Gottschling (2006)
Aquilegia (Ranunculaceae)	25/70	No	Ancestral pollination by bees with two transitions to pollination by hummingbirds and five transitions from hummingbird to hawkmoth pollination	Whittall and Hodges (2007)
Penstemon (Plantaginaceae)	194/270	No	Ancestral pollination by bees with at least 13 shifts to hummingbird pollination	Wilson et al. (2006)
Penstemon (Plantaginaceae)	163/271	No	Ancestral pollination by bees with 10 origins of hummingbird pollination, moth-pollinated species also derived	Wolfe et al. (2006)
Ipomoea sect. *Mina* (Convolvulaceae)	6/8	No	Ancestral pollination by bees with one transition to hummingbird pollination	Zufall and Rausher (2004)

outstanding exception is the temperate genus *Penstemon*, which has been the focus of numerous phylogenetic and field-pollination studies.

Our study on *Ruellia* takes into consideration 40–55% of the New World species diversity. *Ruellia* is an especially inviting research system for an additional reason: it is probably young in age (Bremer et al. 2004; Huang et al. 2005; Tripp 2007). The presumed youth of the Acanthaceae including *Ruellia* and related genera implies that there has been less time and therefore less opportunity (compared to many other angiosperms) for extinctions that complicate phylogenetic inferences. We argue that the evolutionary and ecological history of *Ruellia* is saturated with information on pollinator transitions that may be common in angiosperms and especially in the asterid clade, and that the genus is a clear candidate for research into floral diversification. To our knowledge, this study represents the most taxonomically comprehensive investigation of pollination system evolution in a tropical angiosperm, to date.

Despite innumerable phylogenetic and pollinator studies, we are still lacking adequate data to address questions about floral character change and the evolution of pollination systems. To what extent are flower color transitions unidirectional, and how frequent or labile are they? To better understand floral diversification, we need additional studies on the direction and lability of character transitions in appropriate groups in which powerful, phylogenetic information has been made available (see Armbruster 1996a; Johnson et al. 1998; Givnish et al. 2000; Beardsley et al. 2003; Zufall and Rausher 2004; Pérez et al. 2006). In this study, species-level phylogenetic data are combined with morphological comparative analyses and floral visitor observations to understand the evolution of pollination systems in *Ruellia*. We explicitly test hypotheses about the origins of different pollination systems and provide a critical examination into the lability and directionality of transitions in characters related to pollination. We give special attention to bee/insect and hummingbird-adapted species because they comprise over 70% of our dataset. Specifically, we asked the following questions in *Ruellia*. Have different pollination systems evolved once or multiple times? Are all transitions between pollination systems possible, or does directionality exist as has been previously postulated? To answer these questions, we first assessed whether there were overall differences in floral morphology among species, and whether these differences are associated with particular groups of floral visitors.

Methods

STUDY GROUP

Ruellia is the second largest genus in the Acanthaceae, a family with ca. 4000 species. *Ruellia* is the most diverse in the New World tropics (ca. 275 spp.), particularly in Mexico, Brazil, and western South America. Many species extend into subtropical regions of North and South America, and a few occur in temperate regions. New World species occupy a tremendous breadth of habitats, from high to low elevation rainforests, deciduous forests, savannas, grasslands, deserts, swamps, and bogs. Some species are restricted to endemics pristine habitats while others are common, widespread weeds. In the Old World, approximately 25–35 species occur in Africa (including Madagascar), and a few are distributed from southeast Asia to Australia. Phylogenetic relationships studied by Tripp (2007) indicate *Ruellia* is monophyletic, with Old World taxa forming an early-diverging grade and New World taxa monophyletic and nested within this grade. Most species are herbaceous, although some are shrubs, vines, or lianas.

TAXON AND GENETIC SAMPLING

Our molecular analyses combine previously published data with newly generated sequences. Tripp's (2007) phylogenetic study included sequences for 170 accessions of *Ruellia* from two nucleotide regions: the nuclear ribosomal ITS + 5.8S and the chloroplast *trnG-trnR* regions. We reduced this dataset to 116 taxa: 115 species of *Ruellia* and one outgroup, *Sanchezia speciosa*. These 115 taxa were drawn from all the major lineages of New World *Ruellia* in proportion to the extent of their morphological variation. Only three of these 115 taxa are Old World species: they were included primarily to serve as placeholders for early diverging lineages within the genus. We focused on New World species of *Ruellia* because they are monophyletic and because Old World taxa are poorly known to us. This 116-taxon dataset is the matrix we use for the majority of our analyses. We also conducted a sensitivity analysis to assess whether ancestral reconstructions would change, or how they would change, if we were to consider a larger sampling of the species diversity. For this, preliminary analyses of a 154-taxon matrix were conducted using only ITS + 5.8S sequences. Taxon voucher and GenBank accession numbers are provided in Appendix 1.

Because several lineages were weakly supported in Tripp's study (2007), we added a third nucleotide region to the dataset to improve overall resolution and branch support. The chloroplast *trnG-trnS* spacer was successfully amplified for 70 of the 116 taxa. We used primers described by Hamilton (1999). This region has been shown to be variable in angiosperms as well as within Acanthaceae (McDade et al. 2005; Kiel et al. 2006). The *trnG-trnS* region in *Ruellia* is approximately 860 bp.

DNA EXTRACTION AND AMPLIFICATION

Protocol for the extraction and amplification of DNA follows that of chloroplast data from Tripp (2007). Sequences were generated on an ABI 3730-XL capillary sequencer (Applied Biosystems, Foster City, CA) and assembled using Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI). Sequences were manually aligned using MacClade version 4.06 (Maddison and Maddison 2003). Alignments and trees have been deposited in TreeBASE (Accession No. S1980).

PHYLOGENETIC ANALYSES

We analyzed the 116 taxa, combined data matrix using Bayesian and Maximum-likelihood (ML) methods with MrBayes version 3.06 (Huelsenbeck and Ronquist 2001) and GARLI (Zwickl 2006). Ambiguous alignment sites were excluded. ModelTest version 3.7 (Posada and Crandall 1998) and the Akaike information criterion were used to select a GTR + I + Γ model of sequence evolution for the Bayesian analysis. Two independent Bayesian analyses were run for one million generations each, with sampling every 50 generations. A majority rule tree was constructed in PAUP* (Swofford 2002) using the last 500 trees from each run, excluding pre burn-in trees (we note that the Bayesian MR topology is stable whether built using 1000 or 10,000 trees). The GARLI ML analysis used a model of evolution with four gamma distributed rate categories and an estimated alpha parameter with invariable sites. The run was terminated after 121,500 generations. Branch support was assessed using Bayesian posterior probabilities and an ML bootstrap (100 replicates, 28,700 generations). Bayesian analyses were conducted to examine the effects of missing *trnG-trnS* data by reducing our matrix to the 70 taxa that contained sequences from all three regions. Finally, we conducted Bayesian analyses on the expanded 154-taxon dataset with ITS sequences only. Search conditions for both the 70-taxon *trnG-trnS* and 154-taxon ITS analyses followed those of the 116-taxon analysis above (i.e., same model of evolution, number of generations, majority rule of the last 10,000 trees.

PHYLOGENETIC HYPOTHESIS TESTING

We tested four alternative phylogenetic hypotheses that (1) all purple-flowered, (2) red-flowered, (3) yellow-flowered, and (4) white-flowered taxa are monophyletic. Topologies were constructed in MacClade version 4.06 (Maddison and Maddison 2003) and GARLI (default settings) was used to find the most likely tree consistent with these constraints. Because the K-H likelihood test can be misleading when comparing a priori to a posteriori trees (Goldman et al. 2000; Buckley et al. 2001), we used a one-tailed Shimodaira-Hasegawa (1999) test (RELL optimization, 100 bootstrap replicates) and compared our most likely, unconstrained tree to our most likely, constrained tree (both derived from GARLI).

MORPHOLOGICAL ANALYSES

Fourteen morphological traits were measured for all 116 taxa, with exception of three purple-flowered taxa (*Ruellia discifolia*, *R. insignis*, and *R. pygmaea*) that lacked corollas (Fig. 1; Appendix 2). Of these, nine were floral traits, four were inflorescence traits, and one was a nonfloral trait. These traits were: corolla tube (narrow, unexpanded portion of corolla) length, corolla tube width, corolla throat (expanded portion of corolla) length, corolla throat width, corolla lobe length, corolla lobe width, corolla lobes reflexed or not, corolla color at anthesis, length of stamen exsertion (measured from mouth of corolla opening to the most proximal portion of the most exserted anther), peduncle + pedicel length (or if flowers sessile but part of an inflorescence, simply peduncle length), peduncle thickness (measured at the thickest point), inflorescences axillary or terminal (note that some species of *Ruellia* characteristically produce flowers both in leaf axils and in axils of upper, reduced leaves such that they appear "pseudo-terminal; plants of this type were scored as having axillary inflorescences), whether flowers were substantially protruding beyond vegetative portions of the plant (as a measure of how unobstructed flowers are for pollinator access), and plant height (< 2 m, ≥ 2–2.99 m, ≥ 3 m or plants climbers, i.e., vines or lianas). The first six corolla measurements, length of stamen exsertion, peduncle + pedicel length, and peduncle thickness were continuous measurements; the remainder were discrete. Final measurements were taken from only one specimen per species after a pilot study (E. Tripp, unpubl. data) suggested intraspecific variation was minimal.

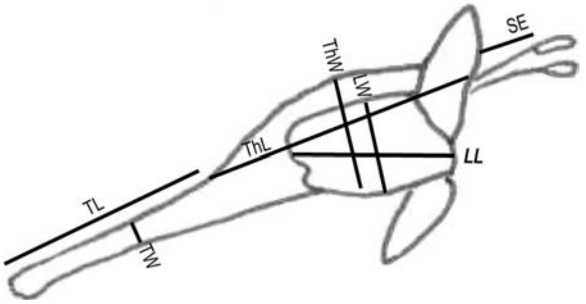

Figure 1. Corolla of *Ruellia asperula* used to show how measurements were made for morphological characters. Character abbreviations follow those in Appendix 1: TL, corolla tube length; TW, corolla tube width; ThL, corolla throat length; ThW, corolla throat width; LL, corolla lobe length; LW, corolla lobe width; SE, length of stamen exsertion. See methods for additional details. For this species, lobes were scored as reflexed. Continuous characters not shown include peduncle + pedicel length and peduncle thickness.

Corolla color was assigned to one of four states: purple, red, white, or yellow to green. A few species have corolla colors that are intermediate between these states. To simplify character evolution analyses, these intermediates were classified into the best-fitting color category. These codings (with actual corolla color in parentheses) are: *Ruellia amoena* (fuchsia) = red; *R. biolleyi* (purplish-white) = purple; *R. chartacea* (reddish-orange) = red; *R. eumorphantha* (purplish-red) = red; *R. floribunda* (magenta) = red.

We used principal components analyses (PCA) to assess whether there were general differences in floral morphology among species of *Ruellia*. PCAs were used over alternative methods, e.g., Discriminant Function Analyses, because PCAs do not identify categories a priori. Two different PCAs on correlations were conducted: one on only red- and purple-flowered taxa because these two states comprise 85 of the 116 taxa, and the second on all four colors. The PCA variables were the nine continuous measurements from above. These characters are important in the attraction and efficacy of pollinators. We also used analysis of variances (ANOVAs) (implemented in statistical package JMP ver. 6, SAS Institute Inc., Cary, NC) to first determine if there were significant morphological differences among the four modes of pollination. If significant differences were found, pairwise Tukey tests were used to determine which pollination system(s) was driving the overall difference. We did not employ methods that correct for phylogeny because we were not able to locate appropriate software to conduct tests between one continuous and one discrete character. No attempts were made to bin continuous data into discrete categories because this results in a loss of information and flower shape is likely discontinuous only at coarser levels (e.g., radial vs. bilateral summetry).

FLORAL VISITORS

We asked whether differences in floral morphology were, by our best estimate, associated with different presumed plant pollinators. We compiled a list of known floral visitors to species of *Ruellia*. These data are based on a literature survey, notes from herbarium labels, personal communications, and personal observations made by the first author over 40 cumulative hours. Table 2 provides information and references for a total of 26 species with known visitors. We included nine additional species for which reasonable inferences regarding pollinators have been made in the literature or by observation (these nine denoted with an asterisk), for a total of 35 species. We note corolla color in the table because it is among the most important and frequently used characters in characterizing pollination systems. Not all of species in Table 2 were included in phylogenetic analyses. We recognize two shortcomings of our study: that we lack data on which visitors are effective pollinators, and present a simplified view of pollinator behavior. Namely, we give little attention to certain traits that are meaningful to attracting animal visitors, for example, quantity, quality, and accessibility of nectar, pollen protein content, etc. However, we are not yet at a stage in *Ruellia* where we can incorporate such traits and pollinator dynamics into a large-scale analysis. Instead, we present data on known floral visitors to support our pollinator behavioral assumptions. By developing new and testable hypotheses, we hope that our study inspires follow-up research on this engaging research system.

We tested for an association between floral color and visitor(s) using a chi-squared test. If a species was polymorphic for a visitor, for example, *Ruellia californica* is visited by both bees and hummingbirds, it was included twice in the analysis (once for purple flowers and bees, once for purple and hummingbirds).

ANCESTRAL STATE RECONSTRUCTIONS

We reconstructed ancestral states using maximum likelihood in Mesquite (Maddison and Maddison 2006) for corolla color. Reconstructions were made on supported nodes over a posterior distribution of trees from the Bayesian analyses (the composite last $500 \times 2 = 1000$ trees), thus taking phylogenetic uncertainty into account (Arnold et al., in press; Reeb et al. 2004; Leschen and Buckley 2007). We used a one-parameter (MK1) instead of a two-parameter model of evolution because corolla color is a multistate character (Maddison and Maddison 2005). To test the robustness of our multistate character reconstructions, additional reconstructions were conducted on each of e four colors coded as binary characters (e.g., purple/not purple). Pie charts that represent relative proportion(s) of reconstructed states across the 1000 trees were generated with precision using the Pie Graph Tool in Adobe Illustrator CS2 (Adobe Systems Incorporated, San Jose, CA).

The process of ancestral state reconstruction results in one or more character states being significantly reconstructed as ancestral for a given node (or if no state is significantly more likely than the others, the reconstruction is equivocal). For example, for a character with two states, the most likely ancestral state for a given node is found by separately maximizing the likelihood of each alternative state (using two different estimates of transition rate parameters), and then comparing the likelihoods using the Likelihood Ratio statistic $\mathrm{LR} = -2\log_e$ (smaller likelihood/larger likelihood). Because the two competing hypotheses (e.g., the likelihood of state 0 vs. likelihood of state 1) are not nested, the LR logarithm does not always follow a chi-squared distribution and so a difference of two log units is interpreted as a “measure of support” and is used in place of a P-value (Pagel 1994).

To estimate the relative transition rates between the four pollination systems, we used a Bayesian stochastic character mapping approach (Nielsen 2001; Huelsenbeck et al. 2003) implemented in SIMMAP 1.0 (Bollback 2006). The program simulates ancestral states over a distribution of trees by sampling characters histories in proportion to their posterior probabilities. We used an $Nst = 1$ model of evolution (corresponding to the MK1 model in MESQUITE) and a fixed prior on the bias parameter to simulate histories over our 1000 Bayesian trees. We did three realizations per tree for a total of 3000 simulated character histories. The Bayesian posterior expectation for each rate is the mean rate per tree averaged over all simulations.

MORPHOLOGICAL EVOLUTION

Results from our ancestral reconstructions prompted specific questions on the evolution of floral traits between bee and hummingbird flowers. Can we detect any historical signature in the

Table 2. Observations and references to known floral visitors/pollinators to species of *Ruellia*. An asterisk indicates pollinator has not been physically seen but strong inferences have been made in the literature or by observation.

Plant species	Corolla color	Location	Pollinator(s)	Reference(s)
Ruellia asperula	Red	Brazil	Hummingbirds	Machado and Sazima (1995)
Ruellia biolleyi	Purple	Costa Rica	Trigonid bees	O. Vargas (pers. obs.)
Ruellia bourgaei	Yellow	Mexico	Bats	Chávez (1974), Ramamoorthy (1991)
Ruellia brevifolia	Red	Brazil	Hummingbirds and Heliconius butterflies	Sigrist and Sazima (2002)
Ruellia californica	Purple	Sonora and Baja Sur, Mexico; Arizona	Bees and hummingbirds	Daniel (2004, 1997), L. A. McDade (pers. obs.), Freeman (1986)
Ruellia caroliniensis	Purple	North Carolina, Florida	Bees, wide variety of flying insects	E. A. Tripp (pers. obs., #114, DUKE), Long (1971a)
*Ruellia ciliatiflora**	Purple	Bolivia and subtropical South America	Bees	Ezcurra and Azuke (1989), Wasshausen and Wood (2003)
Ruellia coccinea	Red	Puerto Rico	Hummingbirds	Kodric-Brown et al. (1984)
*Ruellia exostemma**	Yellow	Venezuela	Bats	Vogel et al. (2004)
*Ruellia eurycodon**	Yellow	Brazil	Bats	Vogel et al. (2004)
Ruellia foetida	White	Guerrero and Oaxaca, Mexico	Butterflies	E. A. Tripp (pers. obs., #185, DUKE), Daniel (1990)
Ruellia fulgens	Red	French Guiana	Hummingbirds	Gracie (1991)
Ruellia galeottii	Purple	Oaxaca, Mexico	Bees	E. A. Tripp (pers. obs., #187, DUKE)
Ruellia geminiflora	Purple	Venezuela	Bees	Ramirez (2004)
Ruellia haenkeana	Red	Bolivia	Hummingbirds	D. C. Wasshausen (pers. obs.)
Ruellia humboldtiana	Red	Venezuela	Hummingbirds	Daniel (1990), herbarium label (Skinner 11, DUKE)
Ruellia humilis	Purple	Pennsylvania	Bees, butterflies, diurnal moths	E. A. Tripp (pers. obs.; #14, DUKE), Estes and Thorp (1975), Tripp (2004)
Ruellia inflata	Red	Bolivia	Hummingbirds (and pollen-robbed by Trigonid bees)	A. Schmidt-Lebuhn (pers. obs.)
Ruellia jaliscana	Yellow	Jalisco, Mexico	Hummingbirds	E. A. Tripp (pers. obs. #199, DUKE)
Ruellia lactea	Purple	Puebla, Mexico	Bees	E. A. Tripp (pers. obs. #164, DUKE)
Ruellia macrophylla	Red	Venezuela	Hummingbirds	Herbarium label (McDade 613, DUKE)
*Ruellia macrosolen**	White	Bolivia, Paraguay, Argentina	Nocturnal lepidopterans	Ezcurra and Azuke (1989)
*Ruellia malaca**	Yellow	Venezuela	Bats	Vogel et al. (2004)
Ruellia malacosperma	Purple	Oaxaca, Mexico	Bees	E. A. Tripp (pers. obs.)
*Ruellia megachlamys**	White	Ethiopia	Long-tongued moths	E. A. Tripp (pers. obs., narrow portions of corolla tube to 130 mm long)
Ruellia nobilis	White	Bolivia	Nocturnal moths	Wasshausen and Wood (2004)
Ruellia noctiflora	White	Florida	Nocturnal moths	Long (1971b)
*Ruelia nocturna**	White	Somalia	Nocturnal moths	Hedrén (1993)
Ruellia nudiflora	Purple	Arizona	Bees, hummingbirds, butterflies, hemiptera	T. F. Daniel (pers. obs.)

Continued.

Table 2. Continued.

Plant species	Corolla color	Location	Pollinator(s)	Reference(s)
Ruellia paniculata	Purple	Costa Rica, Arizona	Bees and butterflies	E. A. Tripp (pers. obs. #122, DUKE); Freeman (1986)
*Ruellia sanguinea**	Red	Argentina, Uruguay, Brazil	Hummingbirds	Ezcurra (1989)
Ruellia speciosa	Yellow	Oaxaca, Mexico	Hummingbirds	E. A. Tripp (pers. obs. #175, DUKE)
Ruellia standleyi	Yellow	Costa Rica	Trigonid bees	E. A. Tripp (pers. obs. (#147, DUKE)
Ruellia sp.	Purple	Oaxaca, Mexico	Small beetles	E. A. Tripp (pers. obs. #174, DUKE)
*Ruellia verbasciformis**	Yellow	Brazil	Bats	Ezcurra and Zappi (1996), Vogel et al. (2004)

morphology of present-day taxa? To attempt to answer this, we tested whether morphological traits differed within a given pollination system, but between clades. In other words, for presumably bee- or insect-pollinated species, we asked whether some trait, for example, corolla tube length, was significantly longer or shorter in clades that were presumably ancestrally hummingbird-pollinated versus clades that were ancestrally bee-pollinated. After comparing traits between clades but within one pollination system, we then asked whether the purple-flowered taxa derived from red-flowered ancestors were statistically more similar to the red flowers in their clade than they were to purple flowers in a different clade. We used trait means of species within a given pollination system to conduct tests. Data were compared to normal distributions then tested for normality using a chi-squared test. Non-normal data were subjected to nonparametric Wilcoxon signed-rank tests whereas normal data were tested with an ANOVA.

Results

PHYLOGENETIC AND CONSTRAINT ANALYSES

The three marker, 70-taxon analysis (with no missing data) resulted in trees with relationships identical to (but less resolved than) those in our 116-taxon analysis. Thus, the incorporation of taxa with missing data had no negative effect, and we emphasize results from the 116-taxon analyses from here forth except where noted otherwise. The ITS + *trnG-trnR* + *trnG-trnS* matrix contained 2849 characters, of which 294 were parsimony informative and 217 were excluded.

The Bayesian Majority Rule and ML ($\ln L = -11743.1$) trees depicted nearly identical relationships among species (Figs. 2 and 3), with the only difference being slightly more resolution in the ML tree. These analyses also recovered the same clades as those in Tripp (2007): the Physiruellia, Euruellia, Ebracteolate, *Blechum*, *R. inflata*, *R. inundata*, *R. harveyana*, *R. humilis*, and *R. jaliscana* clades (Fig. 2). Across the tree, there was moderate clade support ($\geq$ 95% posterior probability (PP) and $\geq$ 70% likelihood bootstrap). Relationships recovered in the full 154-taxon Bayesian analysis (ITS sequences only) reflect those of the 116-taxon analyses. Finally, S-H likelihood tests significantly rejected all alternative phylogenetic hypotheses of purple ($P < 0.05$), red ($P < 0.05$), yellow ($P < 0.05$), and white monophyly ($P < 0.05$).

MORPHOLOGICAL ANALYSES

Using nine morphological variables, the PCA was moderately capable of distinguishing purple from red-flowered taxa (i.e., the majority of species in our dataset, Fig. 4), but was less adept when all four colors were considered (Fig. 5). For the red and purple PCA, the first three PCA axes explained approximately 33%, 25%, and 12% of the variance in the data. All eigenvector coefficients of axis 1 were positive indicating a likely allometric relationship among the variables. For the PCA with all colors, 45%, 21%, and 10% of data variance was explained by the first three axes; similarly, all eigenvector coefficients were positive. Correlations of variables on PCA axes for both analyses are provided in Table 3. For the first PCA, axis 2 positively corresponds to red-flowered taxa and negatively corresponds to purple-flowered taxa. For the second PCA, the first axis best differentiates yellow-flowered taxa from all others. Overall, white-flowered species are morphologically related to species with purple flowers, and yellow-flowered species are most similar to red-flowered species.

The ANOVAs indicated that there were significant differences among the four pollination systems for all traits except corolla lobe length (Table 4; Appendix 2). Bee- or insect-adapted species differed significantly from hummingbird-adapted species in throat length, lobe width, and stamen exsertion, from moth-adapted species in tube length, and from bat-adapted species in tube width, throat width, stamen exsertion, peduncle + pedicle length, and peduncle thickness. Hummingbird-adapted species differed from bee-adapted species in throat length, lobe width, and stamen exsertion as above, from moth-adapted species in tube length, throat length, and lobe width, and from bat-adapted species

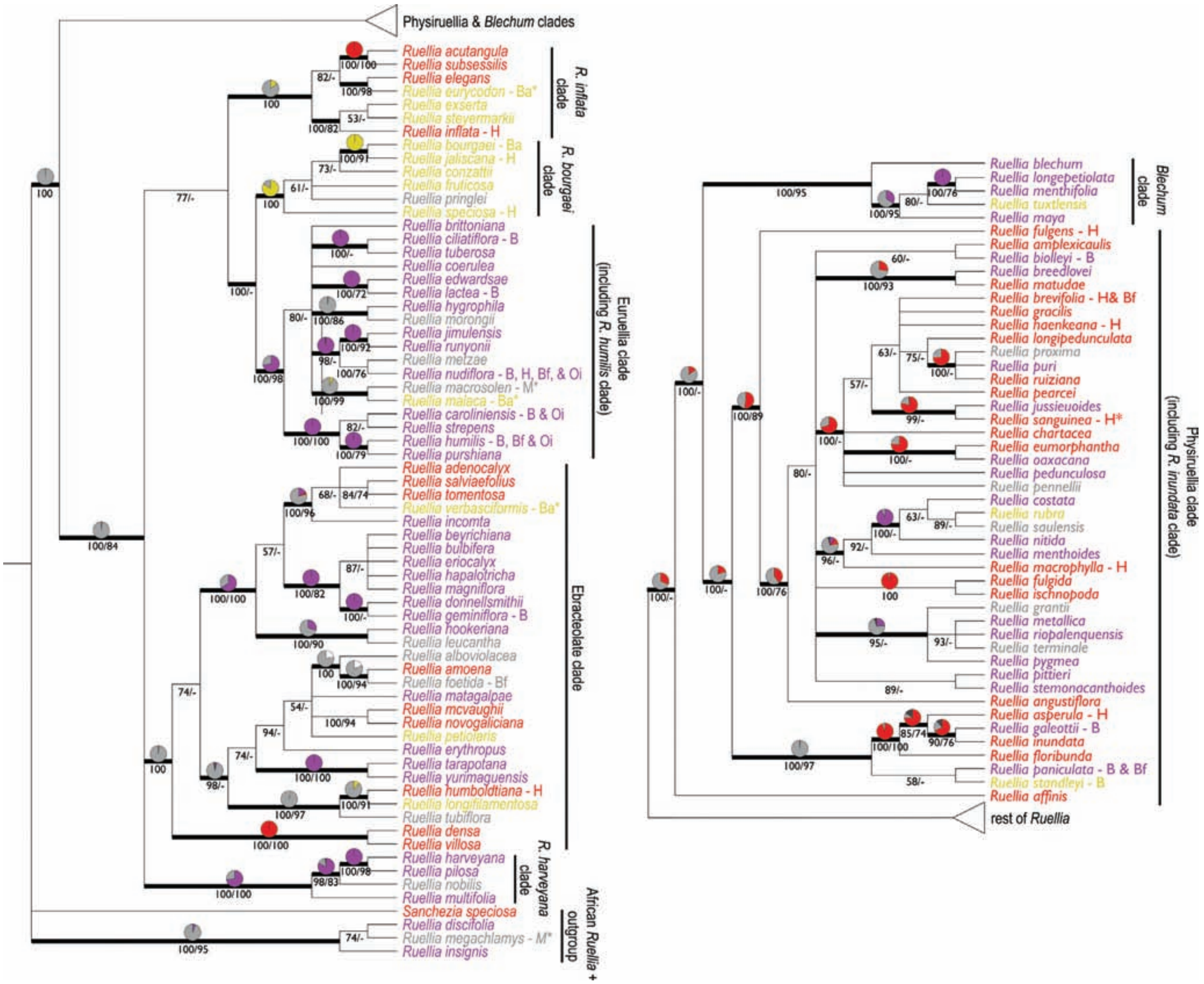

Figure 2. A 116-taxon Bayesian Majority Rule tree showing ancestral reconstructions of corolla color. Thickened branches are supported by either $\geq$ 95% Bayesian posterior probability (below branch to left) and/or $\geq$ 70% ML bootstrap (below branch to right). Major clades of *Ruellia* follow Tripp (2007). Taxon colors correspond to corolla color, with gray used for white-flowered species. Letters to right of taxon names indicate known floral visitors corresponding to Table 2 (B, bee; H, hummingbird; M, nocturnal moth; Ba, bat; Bf, butterfly; Oi, other insect; *, probable pollinator; note that many species have pollinator observations but were not sampled in phylogenetic analysis, see Table 2). Pie charts represent the 1000 Bayesian trees. A colored wedge (purple, red, yellow, and/or white) means that color state(s) was significantly reconstructed as being ancestral in *n* number of the 1000 trees. Gray wedges indicate equivocal reconstruction in that number of trees. Black wedges indicate that node was not present in *n* number of the 1000 trees. The purple- and red-flowered species discussed in the text under "Morphological/Floral Evolution" are seen in the Physiruellia and Euruellia clades.

in tube width, throat width, stamen exsertion, and peduncle thickness. Species with the moth flowers differed from all other groups in tube length, and also from hummingbird-adapted species in throat length and lobe width, and from bat-adapted species in tube width, throat width, stamen exsertion, and peduncle thickness. Finally, bat-adapted species differed from all other groups in tube width, throat width, stamen exsertion, and peduncle thickness. They also differed from bee-adapted species in peduncle + pedicle length and from moth species in tube length.

FLORAL VISITORS

Although a relatively small percentage of the species diversity in *Ruellia* is represented in Table 2, these data in combination with a significant chi-square test ($P < 0.0001$) indicate that corolla color is associated with floral visitors for three of four color categories (excluding the 10 taxa with inferred pollinators, i.e., those with asterisks). All eight species with red corollas were visited by hummingbirds. Of the 11 taxa with purple corollas, 10 were visited by bees and five were also visited by butterflies, diurnal

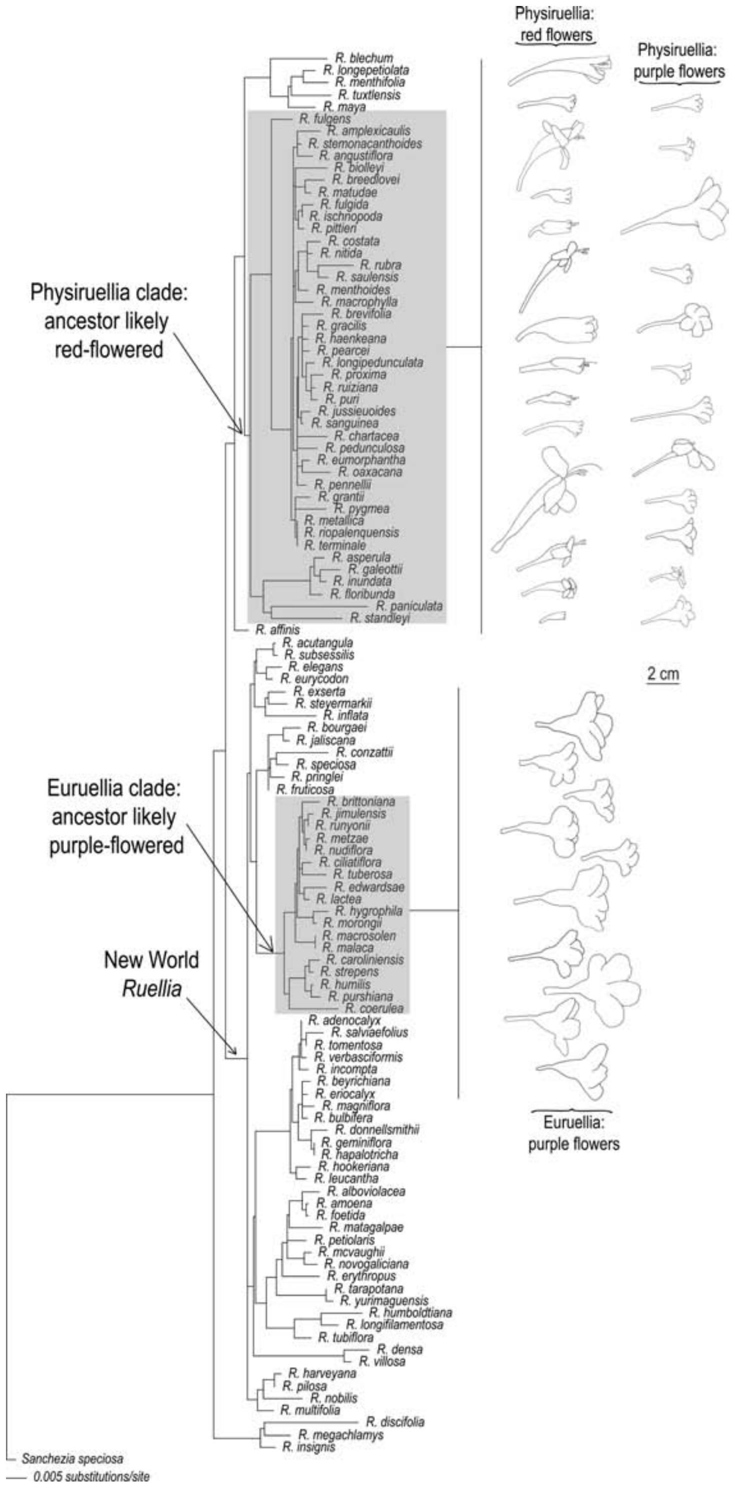

Figure 3. Maximum-likelihood tree showing branch lengths and the evolution of floral morphology in the red-flowered species of Physiruellia, the purple-flowered species derived from red-flowered ancestors in Physiruellia, and the purple-flowered species of the ancestrally purple Euruellia clade. Corollas were drawn from herbarium specimens. They include stamens and/or styles, if visible (i.e., exserted) on herbarium specimens. Scale bar = 2 cm given near drawings. From top to bottom, red-flowered Physiruellia corollas are: *Ruellia amplexicaulis, R. angustiflora, R. macrophylla, R. gracilis, R. brevifolia, R. haenkeana, R. pearcei, R. longipeduncculata, R. ruiziana, R. sanguinea, R. chartacea, R. asperula, R. inundata,* and *R. floribunda*; purple-flowered Physiruellia corollas are: *R. stemonacanthoides, R. biolleyi, R. breedlovei, R. pittieri, R. costata, R. nitida, R. puri, R. riopalenquensis, R. metallica, R. galeottii,* and *R. paniculata*; purple-flowered Euruellia corollas are: *R. brittoniana, R. nudiflora, R. ciliatiflora, R. tuberosa, R. lactea, R. hygrophila, R. caroliniensis, R. strepens, R. humilis,* and *R. coerulea*. See Table 2 for known animal visitors to flowers of some of these species.

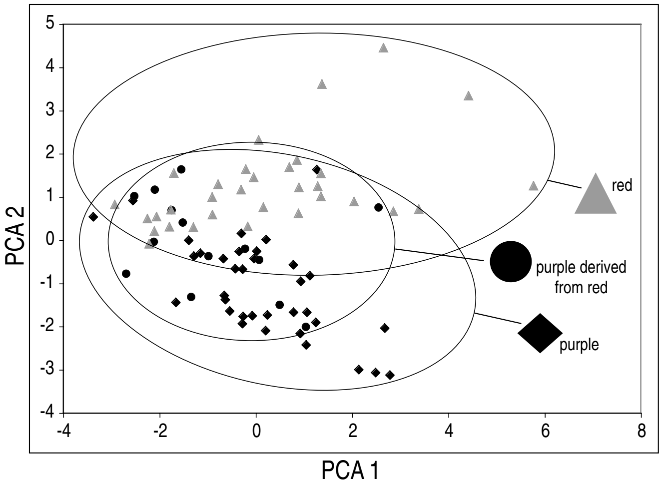

Figure 4. Scatterplot from principal components analysis of purple- and red-flowered New World *Ruellia*. Triangles represent red-flowered species, diamonds represent purple-flowered species, and circles are purple-flowered species in Physiruellia derived from red ancestors of Physiruellia (see captions for Figs. 2 and 3 as well as text for details).

moths, homopterans, or hummingbirds. Two of the three white-flowered species (those with very long corolla tubes) were visited by nocturnal moths; the third, with a narrower and shorter corolla tube, was visited by butterflies. In contrast, yellow-flowered species were visited by a wide variety of animals including hummingbirds, bees, and bats (Fig. 6). Known visitors are also shown in Figure 2.

ANCESTRAL STATE RECONSTRUCTIONS

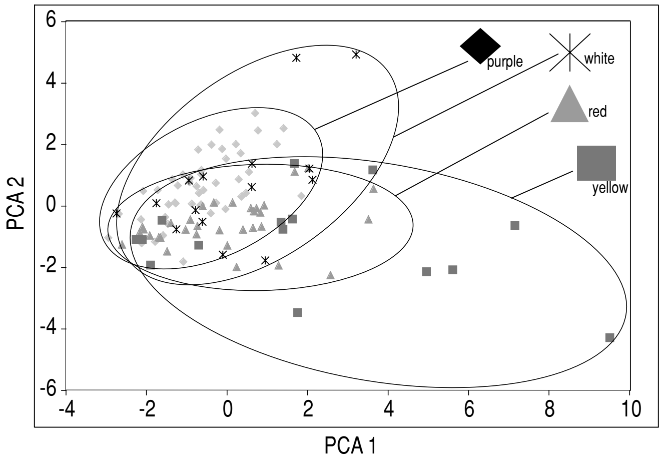

Figure 5. Scatterplot from principal components analysis of all four floral colors. Triangles represent red flowers, diamonds represent purple flowers, squares represent yellow flowers, and asterisk represent white flowers.

Table 3. Eigenvector coefficients for morphological characters used in both PCA analyses; the two-color analysis to the left of slash, and the four-color analysis to the right.

	PCA Axis 1	PCA Axis 2
Peduncle + pedicel length	0.020/0.209	0.478/−0.467
Peduncle thickness	0.159/0.373	0.522/−0.313
Stamen exsertion	0.310/0.402	0.393/−0.138
Corolla tube length	0.321/0.139	−0.215/0.507
Corolla tube width	0.363/0.357	0.125/−0.096
Corolla throat length	0.386/0.329	0.247/ −0.064
Corolla throat width	0.345/0.413	−0.190/−0.093
Corolla lobe length	0.499/0.376	0.157/0.393
Corolla lobe width	0.355/0.301	−0.401/0.482

Figure 2 depicts ancestral state reconstructions of corolla color on supported nodes only. The figure shows multiple transitions among color states. We use an arbitrary ratio of 50% as the cutoff point (i.e., if a given ancestral state was significantly reconstructed in 500 of the 1000 trees) to facilitate our discussion; we also provide both conservative and more liberal estimates of transition numbers based on tree topology. Purple and red flowers served as both ancestral and derived states with respect to other colors. For example, purple is ancestral in one lineage of the Ebracteolate clade and all of the Euruellia clade; in the Physiruellia clade, there are several reverse transitions from red to purple flowers. In contrast, yellow and white flowers were only derived with respect to purple and red (see Physiruellia for example). Of the three largest lineages, red flowers are ancestral in Physiruellia, purple flowers are ancestral in Euruellia, and the ancestral state of Ebracteolate is equivocal. The above pattern were also recovered in the 154-taxon analysis (ITS data only) except that this expanded taxon sampling resulted in some support for purple flowers as ancestral in Ebracteolate (see online Supplementary Fig. S1). Our above inferences did not change when reconstructions on colors coded as binary characters were conducted, thus these data are robust to alternative methods (data not shown). Based on the 116-taxon analysis, the ancestral state(s) of the earliest diverging lineages in *Ruellia* is equivocal. However, in the 154-taxon analysis (see online Supplementary Fig. S1), some of the deepest nodes were reconstructed as purple, suggesting that purple flowers may be ancestral in the genus. Bayesian stochastic character mapping indicated that relative transition rates among the four pollination systems were largely congruent with ancestral reconstructions (Fig. 7). The posterior expectations or means of each rate (and 95% credibility intervals) were purple to red = 7.2 (3–11); purple to yellow = 3.87 (2–6); purple to white = 9.37 (7–12); red to purple = 10.37 (6–14); red to yellow = 3.82 (2–6); red to white = 2.29 (1–4); yellow to purple = 1.78 (0–4); yellow to red = 2.65 (0–5); yellow to white = 2.16 (1–4); white to purple = 1.67 (0–5); white to red = 1.68 (1–3); and white to yellow = 1.98 (0–4).

MORPHOLOGICAL EVOLUTION

For the second part of our morphological analyses (bee or insect vs. hummingbird flowers), there were 14 purple-flowered species in

Table 4. First row shows results of Analyses of Variance to determine whether there were overall trait differences among four pollination systems. Remaining rows show which pollination system(s) is driving overall difference, from pairwise Tukey Tests. Asterisks represent significance ($P<0.05$) and dashes represent nonsignificant values. From left to right, the traits are: corolla tube length and width, corolla throat length and width, corolla lobe length and width, length of stamen exsertion, peduncle + pedicel length, and peduncle thickness.

	Tube ln	Tube wd	Throat ln	Throat wd	Lobe ln	Lobe wd	St exser	Pedu. + pedicel	Ped. thick
ANOVA	$P<0.0005$*	$P<0.0004$*	$P<0.0002$*	$P<0.0002$*	–	$P<0.0008$*	$P<0.0001$*	$P<0.003$*	$P<0.0001$*
BEE × bird	–	–	*	–	–	*	*	–	–
BEE × moth	*	–	–	–	–	–	–	–	–
BEE × bat	–	*	–	*	–	–	*	*	*
BIRD × bee	–	–	*	–	–	*	*	–	–
BIRD × moth	*	–	*	–	–	*	–	–	–
BIRD × bat	–	*	–	*	–	–	*	–	*
MOTH × bee	*	–	–	–	–	–	–	–	–
MOTH × bird	*	–	*	–	–	*	–	–	–
MOTH × bat	*	*	–	*	–		*	–	*
BAT × bee	–	*	–	*	–	–	*	*	*
BAT × bird	–	*	–	*	–	–	*	–	*
BAT × moth	*	*	–	*	–	–	*	–	*

the Euruellia clade that is ancestrally purple and presumably bee-/insect-pollinated (Fig. 2). There were also 14 purple-flowered species and 19 red-flowered species in the Physiruellia clade that is ancestrally red and presumably hummingbird-pollinated (Fig. 2). Purple-flowered taxa that were derived from ancestors with red flowers (Physiruellia) had significantly longer corolla tubes, shorter and narrower throats, and narrower lobes than purple-flowered taxa derived from ancestors with purple flowers (Euruellia) (Table 5). They also tended to have narrower tubes and longer lobes. We then compared corollas of purple-flowered taxa derived from red ancestors (Physiruellia) to corollas of red-flowered taxa within the same clade (Physiruellia). Of the four traits (above) that were significantly different between the purple-flowered taxa of Physiruellia and those of Euruellia, only one differed significantly between red and purple flowers in Physiruellia: corolla throat length.

Discussion

Pollination systems represent an evolutionary specialization that has been important in angiosperm diversification. Data from this study show that there are floral morphological differences among species of *Ruellia* and that these differences are indicative of visitation by particular groups of animals. Floral evolution has been highly labile and also somewhat directional in *Ruellia*. Although some specialized systems such as hawkmoth and bat pollination are probably evolutionary dead-ends, an absolutely striking pattern of reverse transitions is seen in another specialized system. A hummingbird-pollinated lineage has generated as many evolutionary switches to new pollinators as any other ancestral state. Repeated origins of bee- and insect-adapted species from hummingbird-pollinated ancestors occurred through different developmental means, and morphological historical signatures can be seen in the flowers of present-day taxa. As we discuss in closing, some of these pollinator switches may have been adaptive in the evolutionary history of *Ruellia*.

EXISTENCE AND IMPLICATIONS OF FLORAL MORPHOLOGICAL DIFFERENCES

It has been noted that the existence of similar suites of plant reproductive traits in unrelated lineages suggests a certain degree of floral specialization (Wolfe and Sowell 2006). If flowers are indeed specialized, this may be evident on an ordination such that

Table 5. Statistical results from morphological comparison of bee (or other insect) flowers in presumably ancestrally bee-pollinated lineage (Euruellia) with bee flowers in ancestrally hummingbird-pollinated lineage (Physiruellia) and hummingbird flowers in that lineage. See Figure 2 for phylogeny .

	Tube ln	Tube wd	Throat ln	Throat wd	Lobe ln	Lobe wd
Euruellia purple ×	Euruellia longer		Euruellia shorter	Euruellia narrower		Euruellia narrower
Physiruellia purple	$P<0.040$*	$P=0.060$	$P<0.004$*	$P<0.001$*	$P=0.092$	$P<0.030$*
Physiruellia purple ×			Red longer			
Physiruellia red	$P=0.073$		$P<0.0001$*	$P=0.066$		$P=0.054$

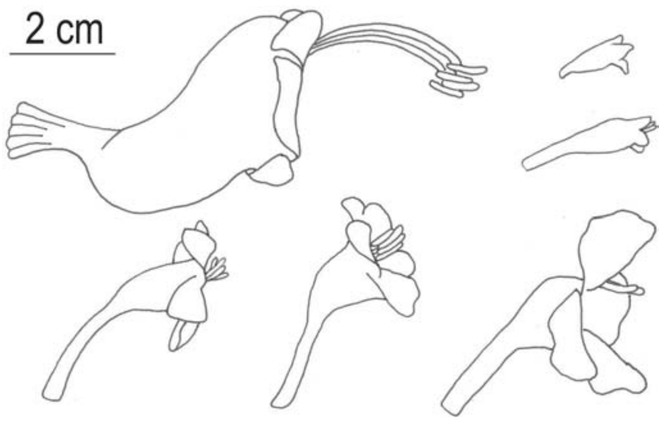

Figure 6. Plate showing morphological diversity of some yellow-flowered species. Moving clockwise starting from largest flower in upper left, corollas (and known floral visitors) of *Ruellia bourgaei* (bats)*, R. standleyi* (bees)*, R. fruticosa* (unknown)*, R. conzattii* (unknown)*, R. jaliscana* (hummingbirds)*,* and *R. speciosa* (hummingbirds). Five of the six species (excluding *R. standleyi*) are monophyletic and endemic to Mexico.

distinct regions of morphospace are occupied by species with similar pollination systems. We addressed this hypothesis using the four major pollination systems of *Ruellia*.

Our PCA results in combination with statistical analyses of traits (see below) show that floral morphology is sometimes indicative of different pollination systems. Similar conclusions have been drawn in other studies (Harrison et al. 1999). PCA clusters were poorer defined in our four-color PCA than they were when we compared only red and purple flowers. This may be the result of the phylogenetically heterogeneous background of species with similar pollination systems. Interestingly, bat- and moth-adapted species tended to occupy morphological extremes on the ordination, with bat flowers most closely resembling hummingbird flowers and moth flowers most closely resembling bee or insect flowers.

Although purple and red flowers were arguable the best differentiated of our four floral colors, these two groups were not as well defined as they have been in other ordinations or discriminant analyses (Harrison et al. 1999; Sakai et al. 1999; Wilson et al.

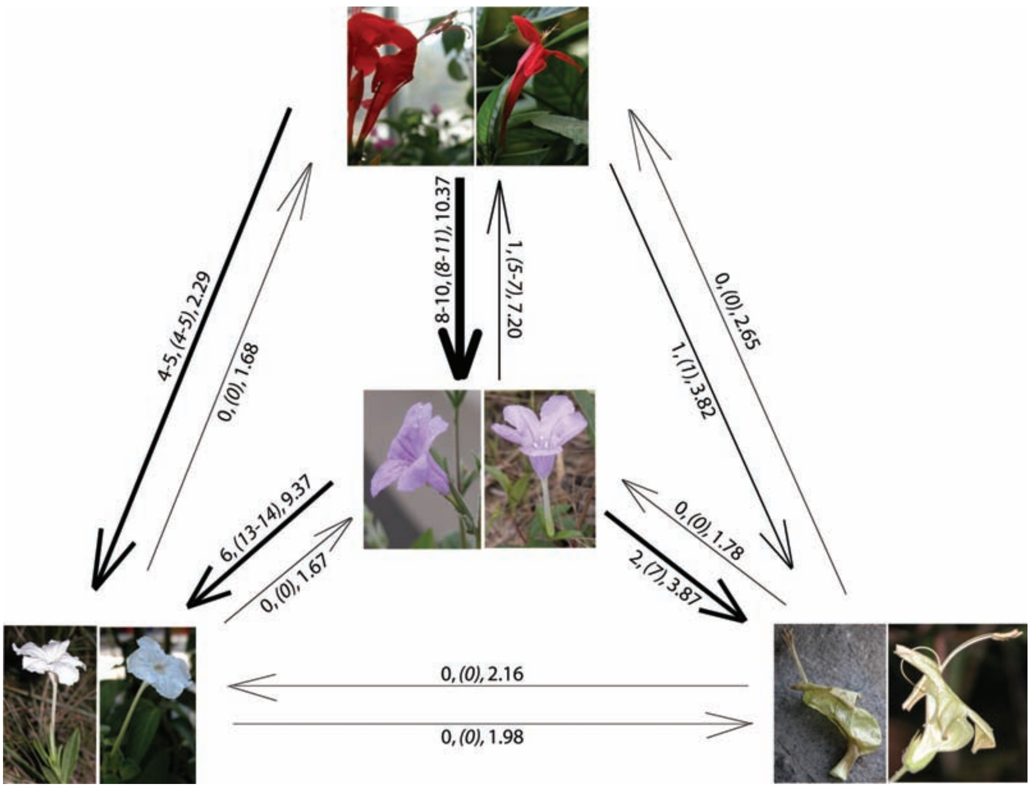

Figure 7. Transitions among pollination systems in *Ruellia*. Direction of arrow represents observed transitions and thickness of arrow represents relative number of transitions based on 116-taxon analysis. Relative number of transitions are provided next to arrows with first set of numbers corresponding to 116-taxon analysis, second numbers (in italics) corresponding to 154-taxon analysis, and third numbers corresponding to Bayesian posterior expectations or transition rates based on stochastic character mapping (95% credibility intervals are provided in text). At the top and moving clockwise, the red-flowered *Ruellia humboldtiana* (left) and *R. mcvaughii* (right) are examples of the hummingbird-adapted flowers, the yellow-flowered *R. bourgaei* (left), and *Ruellia* sp. nov. (right) represent bat-adapted flowers, the purple-flowered *R. nudiflora* (left) and *R. caroliniensis* (right) represent bee- or insect-adapted flowers, and the white-flowered *R. noctiflora* (left) and *R. megachlamys* (right) represent nocturnal moth-adapted flowers.

2004, 2006; Jürgens 2006; Muchhala 2006; Whittall and Hodges 2007). This may also be the result of the heterogeneous backgrounds of red and purple flowers, including the many unorthodox transitions we observe in the genus. Indeed, the datapoints that cloud a clear distinction between red and purple flowers are those purple-flowered species that are derived from red ancestors (see "Reconstructing Pollinator History" and "Floral Evolution" below; Fig. 4).

Statistical analyses more clearly showed that there were significant morphological differences among the four pollination systems in *Ruellia* (see "Results" for details), and that differences largely coincide with traditional conceptions of these systems. These floral morphological differences were also generally associated with different presumed pollinators. All of the red-flowered species were visited by hummingbirds, nearly all of the purple-flowered species were visited by bees (sometimes in addition to hummingbirds or other insects), and most of the white-flowered species were visited by nocturnal moths. The pattern with yellow-flowered species is less clear; for these, corolla shape, timing of anthesis, and other features may be more important to floral visitors than color (Fig. 6). Two yellow-flowered species that are visited by hummingbirds, *R. jaliscana* and *R. speciosa*, have corollas that morphologically resemble some of the red-flowered species, e.g., they have long and slightly narrow tubes. The yellow-flowered, bee-pollinated *Ruellia standleyi* has an oddly shaped corolla that is unlike most other species of *Ruellia*: it has essentially no corolla tube (the narrow, unexpanded portion), but an almost cylindrical throat with very short lobes. The yellow-flowered, bat pollinated species, e.g., *Ruellia bourgaei*, have large, saccate corollas with very wide throats and tubes.

Floral specialization has been discussed under the concept of pollination syndromes (Faegri and van der Pijl 1966; Johnson and Steiner 2000; Fenster et al. 2004). Pollination syndromes are suites of floral traits associated with attracting, utilizing, and reflect adaptations to a particular pollinator or functional group of pollinators (Fenster et al. 2004). Whether syndromes accurately reflect true pollinators is the subject of debate. Several studies have found support for the traditional view that syndromes do reflect pollination by particular animal groups (Schemske and Bradshaw 1999; Borba et al. 2002; Hargreaves et al. 2004; Machado and Vogel 2004; Wilson et al. 2004; SanMartin-Gajardo and Sazima 2005; Muchhala 2006; Pauw 2006; Whittall and Hodges 2007). Other studies, however, have challenged this dogma (Armbruster 1996b; Waser et al. 1996; Olesen and Jordano 2002; Zhang et al. 2005; Linder et al. 2006; Smith et al. 2008). Waser et al. (1996 p. 1053) concluded that generalization in plant-pollinator interactions "appears to be the rule rather than the exception." However, we note that two of the studies (Waser et al. 1996; Olesen and Jordano 2002) supporting this alternative hypothesis focused on broadscale or community-wide patterns, unlike the taxon-specific studies that mostly support the traditional hypothesis. Thus, the conclusions of Waser et al. (1996) and Olesen and Jordano (2002) may be somewhat tangential to our and other studies, although efforts to reconcile potential differences between broad vs. fine scale patterns are desirable.

RECONSTRUCTING POLLINATOR HISTORY

We reconstructed ancestral states of corolla color to assess how many times different pollination systems evolved in *Ruellia*, and whether directionality exists in color transitions. Given that certain corolla colors (Table 2) and shapes (Fig. 3) are associated with a particular floral visitor in *Ruellia* (and in other genera, e.g., *Mimulus*; Bradshaw and Schemske 2003), we used them as a proxy for pollination system.

Data from Figure 2 as well as results from testing alternative phylogenies clearly indicate that different pollinator systems, i.e., bee / insect, hummingbird, bat, and hawkmoth, have all evolved multiple times and that directionality exists in pollinator and color transitions (Fig. 2). When purple flowers were ancestral, we estimate that there were approximately six transitions to white flowers, two transitions to yellow flowers, and only one transition to red flowers. In contrast, when red flowers were ancestral, there were between eight and 10 transitions to purple flowers, four or five to white flowers, and one to yellow flowers. Yellow and white flowers were only derived states. These data strongly support multiple reverse or unorthodox transitions from presumably hummingbird to bee- (or insect-) pollinated species. In *Ruellia*, floral specialization onto hummingbirds is not an evolutionary dead-end.

We explored whether and how our inferences about pollinator transitions would change if we had employed a larger taxon sampling. The pattern of reconstructions from our 154-taxon ITS analysis was similar to what we just described, but as expected we found an overall increase in the number of transitions (see online Supplementary Fig. S1). There were approximately five to seven transitions from purple to red flowers, seven from purple to yellow, and 13 to14 from purple to white flowers. The pattern for red-flowered ancestors was almost exactly the same as our 116-taxon analysis (eight to 11 transitions to purple, four or five to white, and one to yellow). Yellow- and white-flowered species were also only derived with respect to other color states. Thus, we take this repeatability as a measure of support; it increases our confidence in ancestral reconstructions of pollination systems in *Ruellia*.

These results are extraordinary given that nearly all bee or insect / hummingbird studies have shown that hummingbird pollination is derived from bee-pollinated ancestors (Table 1). For example, Stebbins (1989) estimated that the vast majority of the 129 hummingbird-pollinated taxa in the western U. S. A. were derived from insect-pollinated ancestors. Some authors have suggested a possible explanation for this pattern (Thomson et al. 2000;

Castellanos et al. 2004). After evolutionary changes in pollen presentation (e.g., unrestricted opening towards a gradual opening of anthers) in addition to other "anti-bee" and "pro-bird" adaptations, a switch from hummingbird to bee pollination becomes very unlikely because hummingbirds probably transfer pollen much more efficiently than bees, and bee visits essentially become parasitic. Yet we still lack a mechanistic explanation for why there are so many reverse transitions in *Ruellia*.

While both red and purple flowers served as ancestral states with respect to other colors, the pattern of evolution in yellow- and white-flowered taxa, several of which are presumably bat- or hawkmoth-pollinated, is markedly different. We find very little support and very reduced transition rates for changes from yellow or white flowers to purple or red flowers. Species that have dramatically reduced or have lost their floral pigmentation are probably evolutionary dead-ends with respect to new pollinator systems. Thus, our data are mostly consistent with Dollo's Law (Gould 1970). Once a character state is lost, here, red or purple floral pigmentation, it cannot be regained. Other studies have also shown that greenish-yellow or white flowered species are only derived (Perret et al. 2003; Pérez et al. 2006; Whittall and Hodges 2007). There are, however, alternative hypotheses consistent with the data that were not tested by us. One possibility is that our sample size for yellow and white-flowered taxa is too small to recover transitions in other directions. However, over 30 species of *Ruellia* in the 116-taxon analysis are either yellow or white, thus we would expect at least one to a few transitions if such were possible. Another possibility is that, assuming Acanthaceae and *Ruellia* are relatively recent in origin, bat- and hawkmoth-pollinated species may reflect very recent adaptations that have not had sufficient time for further evolutionary change.

One plausible explanation for this pattern in *Ruellia*, as well as in other angiosperms, might be irreversible changes in an underlying pigment pathway. For example, Zufall and Rausher (2004) showed that in the red-flowered lineage *Ipomoea* sect. *Mina*, the loss of ability to produce purple anthocyanins (cyanidins) and subsequent relaxed selection on genes in the purple pigment pathway make future production of purple flowers very unlikely in this group. Thus, the evolution of new pollination systems that involve a loss of ability to produce floral pigments, such as the yellow- or white-flowered species of *Ruellia*, may constrain further evolution toward different pollination systems. We are suggesting that certain types of plant adaptations such as transitions to new pollinators can be, and may frequently be, evolutionary dead-ends with respect to other pollination systems.

Our data support the growing conclusion that recurrent pollinator switches among closely related species have been widespread in angiosperms (Armbruster 1993; Johnson et al. 1998). An interesting perspective that is not necessarily exclusive of this conclusion was recently addressed by Wilson et al. (2006). They discussed "conservatism" or stasis in pollination systems of *Penstemon*. Despite the numerous transitions from bee to hummingbird pollination and dramatic degree of convergent evolution, the authors observed that most branches in the phylogeny of *Penstemon* were still unassociated with pollinator shifts. In *Ruellia*, the degree of convergent evolution is apparently more extreme than in *Penstemon*. Can this discrepancy be explained by the temperate versus tropical nature of these two lineages, or by greater pollinator diversity in one region over the other?

FLORAL EVOLUTION

Data from our comparison of bee versus hummingbird flowers in the Euruellia and Physiruellia clades support Stebbins' (1970) keen insight into trends in the morphological evolution of flowers. He noted (pp. 321–322) that when reversals in environmental conditions prompt evolutionary reversals, ". . .they do not retrace the original evolutionary pathway with respect to the details of the structure, but only with respect to general adaptation." We demonstrated that flowers from bee- or insect-pollinated taxa that were derived from hummingbird-pollinated ancestors (Physiruellia) are morphologically different from the bee-pollinated flowers of another clade (Euruellia) that is ancestrally bee pollinated. In fact, the bee flowers in Physiruellia resemble the hummingbird flowers in Physiruellia to a greater degree than do the bee flowers in Euruellia. Bee flowers in Physiruellia have corollas with significantly longer tubes, shorter and narrower throats, and narrower lobes than bee flowers in Euruellia (Fig. 3). These results indicate that there are multiple means of achieving a similar end functionally (McDade 1992) and that such transitions have not been free of historical baggage: evidence of hummingbird-pollinated ancestry is reflected in modern day corolla morphology. Our results indicate one thing further: seemingly unlikely evolutionary transitions, for example, switches from specialized to more generalized pollination systems, are easier to reconcile if we identify homologies in morphological evolution.

ARE POLLINATOR TRANSITIONS ADAPTIVE?

Evolutionary change in floral morphology may result from historical events or random processes that were unrelated to adaptations to plant pollinators (Armbruster 1996b). Alternatively, it may be driven by selection pressures imposed by pollinators (Hapeman and Inoue 1997; Johnson et al. 1998; Pérez et al. 2006). That is, change may be adaptive. Differentiating between these two hypotheses requires a historical perspective such as a phylogeny. If floral changes were adaptive, we would expect them to occur concomitant with switches to new pollinators. We mapped presumed pollinators onto the ancestral state reconstructions from Figure 2. There were four instances where changes in floral morphology coincided with the predicted change to new presumed pollinators. A switch from red to purple flowers of *R. biolleyi,* red to purple flowers of *R. galeottii*, and red to purple flowers of *R. paniculata* coincided with switches from hummingbird visitation to visitation by bees and butterflies. A switch from purple to white flowers of *R. nobilis* coincided with a transition to visitation by nocturnal moths. Thus, our data are consistent with the hypothesis that some floral diversification events in *Ruellia* can be attributed to pollinator transitions. But have pollinator isolating mechanisms contributed to reproductive isolation in the genus (Ramsey et al. 2003)? The importance of pollinator isolation could be addressed by examining sympatric sister species that differ in their pollinators. Although data on the frequency of sympatric sister species are scant in *Ruellia* (but see Ezcurra and Azuke 1989 for an example), Figure 2 shows that there are approximately equal numbers of sister species pairs with similar pollination systems as there are species pairs with different systems (17 to 15). Of 32 (total) sister species pairs in the phylogeny, over three-quarters of them contain species with similar geographic ranges, and these sister species may well be (or have been historically) sympatric in some part of them. This interesting system lends itself to further study.

CONCLUSIONS

Our results contribute new information to the debate about whether specialization is an evolutionary dead-end. We learned that rather than simply querying "are specializations evolutionary dead-ends," a more productive approach might be to ask, for a given group of organisms (Futuyma and Moreno 1988), and a particular class of adaptations, "just how much specialization does it take to arrive at a dead-end?" In other words, what types of transitions are dead-ends? Data presented here indicate that loss of some corolla pigments (e.g., anthocyanins) is likely irreversible in *Ruellia*, and that adaptation to pollination by bats and hawkmoths may be a dead-end with respect to the potential for exploiting new pollinators. Conversely, we have shown bidirectional transitions between bee- and hummingbird-pollinated species, and that evolutionary specialization to hummingbird pollination is clearly not a dead-end in *Ruellia*. Yet why are there so many reverse transitions in *Ruellia*? Perhaps the most likely explanation is repeated dispersal to habitats that lack hummingbirds. Under such a scenario in which selective pressures to maintain adaptation to bird pollination have been lifted, bee or other modes of pollination may be free to evolve. Assuming no major range shifts on behalf of the interacting species, this hypothesis is presumably testable by overlaying habitat and/or hummingbird distribution maps onto the clade containing both hummingbird-adapted and hummingbird-derived, bee-adapted species. Similarly, the repeated evolution of hummingbird pollination in *Ruellia* might be explained by multiple dispersals to hummingbird-rich environments, particularly in western South America where the diversity of hummingbirds is extraordinary (Bleiweiss 1998). The apparent higher degree of pollinator lability in *Ruellia* than in other groups such as *Penstemon* may relate to a greater number of biotic interactions in tropical over temperate biomes, although this remains to be tested.

Pollination systems are indicative of a term that has permeated the biological sciences: specialization. Biologists have viewed floral specialization as being omnipresent or conversely, of little importance. This dichotomy is largely due to a lack of information about a complex relationship that represents the culmination of pollinator behavioral preferences, the actions of other important symbionts such as floral antagonists, dynamics of individual flowers within a species, and effective plant pollination (see Castellanos et al. 2003; McDade and Weeks 2004; Richardson 2004; and Gegear and Burns 2007 for examples of this complexity). Unfortunately, even the most basic information such as whether presumed pollinators are actually doing the pollinating is largely lacking for most animal-pollinated plants. Broad-scale studies are desirable because they identify smaller, targeted lineages in which interesting hypotheses can be tested through careful field studies. We concur with Johnson and Steiner (2000, p. 143) that pollination systems are "sorely in need of more critical examination" by conducting field experiments. In addition, we can make many more inferences and derive much more powerful results if we do so in lineages in which near-complete, species-level phylogenies have been made available. *Ruellia* is a candidate system for such research.

ACKNOWLEDGMENTS

We thank L. Bukovnik from the Duke Biology Sequencing Lab, T. Daniel, L. McDade, A. Schmidt-Lebuhn, O. Vargas, and D. Wasshausen for pollinator observations; K. Deregibus for preparing the corolla drawings in Figures 3 and 5; R. Gegear, one anonymous reviewer, F, Lutzoni, L. McDade, M. Rausher, J. Shaw, and the Manos lab group for their thoughtful comments. An American Philosophical Society Lewis & Clark grant to EAT; and a DDIG grant (NSF DEB-0608363) to PSM and EAT supported this research.

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Associate Editor: J. Shykoff

Appendix 1:

Taxa, voucher information, and GenBank accession numbers (ITS, *trnG-trnR*, *trnG-trnS*) for 116 species used in this study. Note that there are 23 species of *Ruellia* included in the 154-taxon analysis (online Supplementary Fig. S1) that were not part of Tripp's (2007) study. These taxa are not included in the Appendix below because some species identifications are still tentative, and these taxa are part of other analyses in progress; thus, we consider them unpublished data. These 23 include three species currently recognized as belonging to *Blechum,* and two species of *Blechum* that were recently transferred to or re-established as species of *Ruellia* (McDade and Tripp 2007). However, the *Blechum* lineage is clearly nested within *Ruellia* and a forthcoming manuscript places the entire genus into synonymy with *Ruellia* (Tripp et al., in review).

Ruellia acutangula (Nees) Lindau—*Folli 3585* (US), Brazil, (EF214437, EF214626, EU431011); *Ruellia adenocalyx* Lindau—*Aparecida & al. 5047* (US), Brazil, (EF214438, EF214627, EU431012); *Ruellia affinis* (Nees) Lindau—*Carvalho & al. 6006* (US), Brazil, (EF214439, EF214628, EU431013); *Ruellia alboviolacea* Lindau—*Hinton 11018* (US), Mexico, (EF214440, EF214629, EU431014); *Ruellia amoena* Sessé & Moc.—*Koch & Fryxell 83210* (US), Mexico, (EF214441, EF214631, EU431015); *Ruellia amplexicaulis* (Nees) Lindau—*Kral & Wanderly 75013* (US), Brazil, (ITS: EF214442, trnGR: EF214630); *Ruellia angustiflora* (Nees) Lindau—*Arbo & al. 5922* (US), Argentina, (ITS: EF214445, trnGR: EF214633); *Ruellia asperula* Lindau—*Agra & al. 4777* (MO), Brazil, (ITS: EF214448, trnGR: EF214636); *Ruellia beyrichiana* (Nees) S. Moore—*Gottsberger 30983* (US), Brazil, (EF214449, EF214637, EU431016); *Ruellia biolleyi* Lindau—cult. DUKE greenhouses (EF214450, EF214638, EU431017); *Ruellia blechum* L.—*Sianca-Colín 1914* (MO), Mexico, (EF214412, EF214601 [as *Blechum pyramidatum* (Lam.) Urb.], EU431009); *Ruellia bourgaei* Hemsl.—*Soto-Nuñez & al. 6060* (MO), Mexico, (ITS: EF214452, trnGR: EF214639); *Ruellia breedlovei* T. F. Daniel—*Neill 5560* (MO), Mexico, (ITS: EF214454, trnGS: EU431018); *Ruellia brevifolia* (Pohl) C. Ezcurra—*Neill & al. 11509* (MO), Ecuador, (ITS: EF214455, trnGR: EF214640); *Ruellia brittoniana* Leonard—cult. DUKE greenhouses (wild source: Durham, North Carolina), (EF214458, EF214643, EU431019); *Ruellia bulbifera* Lindau—*Wasshausen & al. 1994* (US), Argentina, (EF214459, EF214644, EU431020); *Ruellia caroliniensis* (Walt.) Steud.—*Wilbur 66082* (DUKE), North Carolina, (ITS: EF214460, trnGR: EF214645); *Ruellia chartacea* (T. Anders.) Wassh.—cult. DUKE greenhouses (source: Fairchild Tropical Gardens), (EF214461, EF214646, EU431021); *Ruellia ciliatiflora* Hook.—*Wood 10383* (US), Bolivia, (EF214463, EF214846, EU431022); *Ruellia coerulea* Morong—*Zardini & Velazquez 23552* (MO), Paraguay, (ITS: EF214465); *Ruellia conzattii* Standley—*Martinez 1770* (MO), Mexico, (ITS: EF214467, trnGS: EU431023); *Ruellia costata* (Nees) Lindau—*Silva 363* (US), Brazil, (ITS: EF214469 trnGS: EU431024); *Ruellia densa* (Nees) Hiern—*Tameirão & Franca 81* (US), Brazil, (ITS: EF214471); *Ruellia discifolia* Oliver—*Boulos s.n.* (MO), Ethiopia, (ITS: EF214475); *Ruellia donnell-smithii* Leonard—*Ventura 925* (DUKE), Mexico, (EF214477, EF214654, EU431025); *Ruellia drummondiana* (Nees) A. Gray – *York 46274* (DUKE), Texas, (ITS: EF214479); *Ruellia edwardsae* Tharp & F. A. Barkley—*Genelle & Fleming 843* (MO), Mexico, (EF214481, EF214656); *Ruellia elegans* Poir.—cult. DUKE greenhouses (source: Marie Selby Greenhouses), (EF214484, EF214659, EU431026); *Ruellia eriocalyx* Glaz.—*Oliveira & Alvarenga 130* (US), Brazil, (EF214485, EF214660, EU431027); *Ruellia erythropus* (Nees) Lindau—*Cristóbal & al. 2282* (MO), Argentina, (ITS: EF214486, trnGR: EF214661); *Ruellia eumorphantha* Lindau—*Acosta 1172* (MO), Mexico, (EF214487, EF214662, EU431028); *Ruellia eurycodon* Lindau—*Costa 38.768* (US), Brazil, (EF214489, EF214664, EU431029); *Ruellia exserta* Wassh. & J. R. I. Wood—*Nave & al. 1581* (US), Brazil (EF214490, EF214665); *Ruellia floribunda* Hook.—*Bonitaz & Cornejo 3612* (US), Ecuador, (EF214492, EF214667, EU431030); *Ruellia foetida* Willd.—*Daniel 2081* (DUKE), Mexico, (EF214493, EF214668, EU431031); *Ruellia fruticosa* Sessé & Moc.—*Thomas 3798* (US), Mexico, (trnGR: EF214670); *Ruellia fulgens* (Bremek.) E. Tripp—*Prance 30665* (US), French Guiana, (ITS: EF214432, trnGR: EF214621); *Ruellia fulgida* Andr.—*Machado 9* (DUKE), Venezuela, (EU431001, EF214671, EU431032); *Ruellia galeottii* Leonard—*Hinton 15323* (PH), Mexico, (ITS: EF214496); *Ruellia geayi* (Benoist) E. Tripp—*Eboroke 811* (MO), Madagascar, (ITS: EF214417); *Ruellia geminiflora* H. B. K.—*Tripp & Dexter 160* (DUKE), Mexico, (ITS: EF214498, trnGR: EF214674); *Ruellia gracilis* Rusby—*Daly & al. 6633* (US), Bolivia, (ITS: EF214499, trnGS: EU431033); *Ruellia grantii* Leonard—*Wood 5221* (US), Colombia, (EF214500, EU431006, EU431034); *Ruellia haenkeana* (Nees) Wassh.—*Smith & Smith 13087* (MO), Bolivia, (ITS: EF214501, trnGS: EU431035); *Ruellia hapalotricha* Lindau—*Macedo 5859* (US), Brazil (ITS: EF214503); *Ruellia harveyana* Stapf—*Monro 2654* (MO), Belize, (ITS: EF214504, trnGS: EU431036); *Ruellia hirsuto-glandulosa* (Oerst.) Hemsl. – *Chiang 2023* (MO), Mexico, (ITS: EF214505); *Ruellia hookeriana* Hemsl.—*Breedlove & Daniel 71041* (US), Mexico (EF214506, EF214677, EU431037); *Ruellia humboldtiana* (Nees) Lindau—*McDade & Lundberg 914* (DUKE), Venezuela, (ITS: EF214507); *Ruellia humilis* Nutt.—*Tripp 14* (PH), Pennsylvania, (EF214508, EF214678, EU431038); *Ruellia hygrophila* Mart.—cult. DUKE greenhouses (source: Kew, coll. from

Argentina), (EF214509, EF214679, EU431039); *Ruellia incomta* (Nees) Lindau—*Mori & al. 16639* (MO), Brazil, (EU431002, EF214510, EU431040); *Ruellia inflata* Rich.—*Gentry & Perry 77992* (MO), Bolivia, (ITS: EF214512); *Ruellia insignis* Balf. f.—cult. DUKE greenhouses (source: Kew, collected from Socotra [Yemen]), (EF214513, EF214680, EU431041); *Ruellia inundata* H. B. K.—*Tripp & Deinert 121* (DUKE), Costa Rica, (ITS: EF214514); *Ruellia ischnopoda* Leonard—*Wood 4757* (US), Colombia (ITS: EF214516, trnGS: EU431042); *Ruellia jaliscana* Standl. *Iltis & al. 29442* (US), Mexico, (ITS: EF214517); *Ruellia jimulensis* Villarreal—*Henrickson & Bekey 18504* (US), Mexico, (EF214518, EF214681, EU431043); *Ruellia jussieuoides* Schltdl. & Cham.—*Tripp & Dexter 156* (DUKE), Mexico, (ITS: EF214519, trnGR: EF214682); *Ruellia lactea* Cav.—*Correll & Johnston 20148* (US), Mexico, (ITS: EF214520, trnGR: EU431007); *Ruellia leucantha* Brandegee—*Carter & al. 5896* (US), Mexico, (EF214522, EF214684, EU431044); *Ruellia longifilamentosa* Lindau—*Gamboa 307* (US), Colombia, (ITS: EF214525); *Ruellia longipedunculata* Lindau—*Wood 13750* (US), Bolivia, (EF214526, EF214686, EU431045); *Ruellia longepetiolata* (Oerst.) Hemsl.—cult. DUKE greenhouses, (ITS: EF214524); *Ruellia macrophylla* Vahl—cult. DUKE greenhouses (source: Kew, coll. from Venezuela), (EF214528, EF214689, EU431046); *Ruellia macrosolen* Lillo ex. C. Ezcurra—*Krapovickas & Cristobal 46267* (US), Argentina (ITS: EF2124529); *Ruellia magniflora* C. Ezcurra—*Wasshausen & al. 2000* (US), Argentina (EF214530, EF214687, EU431047); *Ruellia malaca* Leonard—*Stergios & Delgado 13487* (US), Venezuela, (ITS: EF214531, trnGS: EU431048); *Ruellia matagalpae* Lindau—*Rees & al. 182* (MO), Belize, (EF214533, EF214690, EU431049); *Ruellia matudae* Leonard—*Breedlove & Bourell 67437* (US), Mexico, (EF214535, EF214692, EU431050); *Ruellia maya* T. F. Daniel—*Tripp & Dexter 157* (DUKE), Mexico, (EF214536, EF214693, EU431051); *Ruellia mcvaughii* T. F. Daniel—*Panero & al. 5601* (US), Mexico (ITS: EF214537, trnGR: EF214694); *Ruellia megachlamys* S. Moore—cult. DUKE greenhouses (source: Kew, coll. from Kenya), (EF214539, EF214696, EU431052); *Ruellia menthifolia* Leonard—*Hitchcock & Stanford 6921* (US), Mexico, (ITS: EF214540, trnGR: EF214697); *Ruellia menthoides* (Nees) Hiern—*Foster & al. 110* (MO), Bolivia, (EF214541, EF214698, EU431053); *Ruellia metallica* Leonard—*Tripp & Salazar-Amoretti 148* (DUKE), Costa Rica, (ITS: EU431003, trnGS: EU431054) *Ruellia metzae* Tharp—*Tharp 46054* (DUKE), Texas, (ITS: EF214542, trnGR: EF214699); *Ruellia morongii* Britton—*Zardini & Velazquez 24875* (MO), Paraguay, (ITS: EF214543, trnGR: EF214700); *Ruellia multifolia* (Nees) Lindau—*Zardini 8699* (MO), Paraguay, (ITS: EF214544, trnGR: EF214701); *Ruellia nitida* (Nees) Wassh. & J. R. I. Wood—*Wood 16518* (US), Bolivia, (EF214545, EF214702, EU431055); *Ruellia nobilis* Lindau—*Vargas 3343 & al.* (US), Bolivia, (ITS: EF214546); *Ruellia novogaliciana* T. F. Daniel—*Hinton & al. 12954* (US), Mexico, (EF214547, EF214703, EU431056); *Ruellia nudiflora* (Engelm. & A. Gray) Urb.—*Whitson & Whitson 814* (DUKE), Texas, (ITS: EF214548, trnGS: EU431057); *Ruellia oaxacana* Leonard—*Hinton 15831* (US), Mexico, (ITS: EF214551, trnGR: EF214705); *Ruellia paniculata* L.—*Aymard & Stergios 9546* (US), Venezuela, (ITS: EF214553, trnGS: EU431058); *Ruellia pearcei* Rusby—*Wasshausen & Wood 2139* (US), Bolivia, (EF214557, EF214708, EU431059); *Ruellia pedunculosa* (Nees) Lindau—*Sanchez & Dillon 10194* (US), Peru, (EF214561, EF214712, EU431060); *Ruellia pennellii* Leonard—*Croat 70892* (US), Colombia (EF214558, EF214709, EU431061); *Ruellia petiolaris* (Nees) T. F. Daniel—*Daniel & Bartholomew 4930* (US), Mexico (EF214560, EF214711, EU431062); *Ruellia pilosa* (Nees) Pav. Ex Nees—*Vasquez 434* (MO), Mexico (ITS: EF214562, trnGR: EF214713); *Ruellia pittieri* Lindau—*Croat 22615*, (MO), Costa Rica (ITS: EU431004, trnGS: EU431063); *Ruellia pringlei* Fernald—*Martinez 2053* (MO), Mexico, (ITS: EF214563, trnGR: EF214714); *Ruellia proxima* Lindau—*Killen & al. 2906* (MO), Bolivia, (ITS: EF214564); *Ruellia puri* (Nees) Mart. ex Jackson—*Solomon & Nee 14293* (MO), Bolivia, (ITS: EF214565, trnGR: EF214716); *Ruellia purshiana* Fernald—*Eyles 695* (DUKE), Georgia (USA), (EF214566, EF214717, EU431064); *Ruellia pygmaea* Donn. Sm.—*Contreras 11429* (US), Guatemala, (ITS: EF214567); *Ruellia riopalenquensis* Wassh.—*Webster & Lockwood 22881*, (US), Ecuador (ITS: EF214568); *Ruellia rubra* Aubl.—*Fueillet & al. 10300* (US), French Guiana, (EF214569, EF214718, EU431065); *Ruellia ruiziana* (Nees) Lindau—*Foster 8502* (MO), Peru, (ITS: EF214570, trnGS: EU431066); *Ruellia runyoni* Tharp & F. A. Barkley—*Tharp & Brown 3358* (DUKE), Texas, (EF214571, EF214719, EU431067); *Ruellia salviaefolius* (Nees) Lindau—*Hatschbach & al. 70655* (US), Brazil, (EF214572, EF214720, EU431068); *Ruellia sanguinea* Griseb.—*Wood 1241* (US), Bolivia, (ITS: EF214573, trnGR: EU431008); *Ruellia saulensis* Wassh.—*Granville & al. 14887* (US), French Guiana, (ITS: EF214575, trnGR: EF214722); *Ruellia speciosa* (Nees) Lindau—*Lyonnet 747* (US), Mexico, (EF214576, EF214723, EU431069); *Ruellia spissa* Leonard – *Hinton 1068* (US), Mexico, (ITS: EF214577); *Ruellia standleyi* Leonard—*Tripp & Salazar-Amoretti 147* (DUKE), Costa Rica, (ITS: EF214580); *Ruellia stemonacanthoides* (Oerst.) Hemsl.—*Tripp & Salazar-Amoretti 151* (DUKE), Costa Rica (EF214583, EF214727, EU431070); *Ruellia strepens* L.—*Tripp 25* (PH), Pennsylvania, (EF214585, EF214728, EU431071);

Ruellia steyermarkii Wassh.—*Steyermark 89113* (US), Venezuela, (ITS: EF214582, trnGR: EF214726); *Ruellia subsessilis* (Martius) Lindau—*Vasconcelos s. n.* (US), Brazil, (EF214586, EF214729, EU431072); *Ruellia tarapotana* Lindau—*Smith & al. 941* (US), Peru, (ITS: EF214587, trnGS: EU431073); *Ruellia terminale* (Nees) Wassh.—*Clark 3034* (US), Ecuador, (EF214588, EF214730, EU431074); *Ruellia tomentosa* (Nees) Lindau—*Aparecida & al. 3821* (US), Brazil, (ITS: EF214589, trnGR: EU431075); *Ruellia tuberosa* L.—*Jansen-Jacobs & al. 3869* (US), Guyana, (EF214592, EF214732, EU431076); *Ruellia tubiflora* H. B. K.—*Daniel & al. 6343* (US), Costa Rica, (ITS: EF214591, trnGR: EF214731); *Ruellia tuxtlensis* T. P. Ramamoorthy & Y. Hornelas—*Manriquez & al. 2366* (US), Mexico, (ITS: EF214596, trnGS: EU431077); *Ruellia verbasciformis* (Nees) C. Ezcurra & Zappi—*Mendonca & al. 3519* (US), Brazil, (ITS: EF214597, trnGR: EU431078); *Ruellia villosa* Lindau—*Hatschbach & al. 68100* (US), Brazil, (ITS: EF214598); *Ruellia yurimaguensis* Lindau—*Wood 15005* (US), Bolivia, (ITS: EF214600); *Sanchezia speciosa* Leonard—*Zak 3563A*, (DUKE) Ecuador (GenBank AF169835, EU431005, EU431010).

Appendix 2. Morphological matrix for 116 taxa and 14 traits: Corolla tube length (TL) and tube width (TW), corolla throat length (ThL) and throat width (ThW), corolla lobe length (LL) and lobe width (LW), length of stamen exsertion (SE), peduncle + pedicel length (PL), peduncle thickness (PT), corolla lobes reflexed or not (Lo), corolla color at anthesis (Co), inflorescences axillary or terminal (If), flowers protruding beyond vegetative portions of plant (Pr), and plant height (Ht). Data for first nine traits are continuous and data for the last six are discrete. For Co, P=purple, R=red, Y=yellow, and W=white. For If, Ax=axillary and Te=terminal. For Ht, measurement is in meters, and CL=climbing plant (liana or vine). For all other discrete characters, 1=state present and 0=state absent. All continuous data are measured in millimeters; NA=not applicable; a dash indicates that the datapoint was excluded from analyses because we were not able to score the trait.

	TL	TW	ThL	ThW	LL	LW	SE	PL	PT	Lo	Co	If	Pr	Ht
R. actutangula	12	1.5	24	6	8	8	0	148	0.75	0	R	Ax	1	<2
R. adenocalyx	12	3.5	26.5	8.5	14	8	7.5	50	1	1	R	Te	1	<2
R. affinis	28	3	35	8	20	13	10	3	1.5	1	R	Te	1	CL
R. alboviolacea	16	2	17	10	9	7	0	89	0.75	0	W	Ax	1	<2
R. amoena	13	2	21	5	13.5	7	7	16	1	1	R	Ax	1	<2
R. amplexicaulis	20	1.5	27	7	8	8	3	68	0.75	0	R	Ax	1	<2
R. angustiflora	10	2	24	6	3	2.5	1	58	0.5	0	R	Ax	0	≥2
R. asperula	19	2	16	7.5	7	4	9	42	1	1	R	Ax	1	<2
R. beyrichiana	11	2	20	11	10	13	0	0	NA	0	P	Ax	0	<2
R. biolleyi	10	2	9	9	5	5	0	325	1	0	P	Ax	1	<2
R. blechum	4.5	1	3.5	3	3.5	2	0	22	1	0	P	Ax	0	<2
R. bourgaei	18	7	40	30	20	16	37	792	4.5	1	Y	Te	1	≥2
R. breedlovei	22.5	3	25	13	18	14	0	208	2	0	P	Ax	1	<2
R. brevifolia	6	1	18	11	3	4	0	73	0.75	0	R	Ax	1	≥2
R. brittoniana	11	2.5	19	9	15	15	0	80	1	0	P	Ax	1	<2
R. bulbifera	10	2	16	7	10	13	0	1	1	0	P	Ax	0	<2
R. caroliniensis	20	1.5	11	10	10	8	0	1	0.5	0	P	Ax	0	<2
R. chartacea	10	4	50	10	22	10	14	140	2	1	R	Te	1	<2
R. ciliatiflora	5	2	16	11	10	10	0	83	1.25	0	P	Te	1	<2
R. coerulea	8.5	1	14	8	9	9	0	30	1	0	P	Ax	1	<2

Continued.

Appendix 2. Continued.

	TL	TW	ThL	ThW	LL	LW	SE	PL	PT	Lo	Co	If	Pr	Ht
R. conzattii	20	3	35	23	21	20	2	146	2	1	Y	Te	1	≥2
R. costata	35	1.5	15	8	10	10	0	163	0.75	0	P	Ax	1	<2
R. densa	9	1.5	11	4	1.5	1	0	30	1	0	R	Ax	1	<2
R. discifolia	–	–	–	–	–	–	–	13	1	–	P	Ax	0	<2
R. donnell-smithii	16	2	17	8	9	12	1	1	NA	0	P	Ax	0	<2
R. edwardsae	6	6	23	12	10	10	0	0	NA	0	P	Ax	0	<2
R. elegans	2	2	26	8	15	12	7	151	1	1	R	Ax	1	≥2
R. eriocalyx	10	2	22	14	13	17	0	25	1	0	P	Ax	1	<2
R. erythropus	20	1	10	8	7	6	0	0	NA	0	P	Ax	0	<2
R. eumorphantha	13	2	45	10	12	6	4	144	1	1	R	Ax	1	≥2
R. eurycodon	8	3.5	20	17	11	12	1	182	2	–	Y	Ax	1	CL
R. exserta	10	3	18	12	6	5	11.5	348	4	1	Y	Te	1	CL
R. floribunda	6.5	2	13	6	5	2.5	2	62	0.5	0	R	Ax	0	≥2
R. foetida	17	1.5	8.5	6	7	7	1	20	0.75	0	W	Ax	0	<2
R. fruticosa	13	2.5	11	6	1	1.5	1	39	0.5	0	Y	Ax	0	<2
R. fulgens	25	3	40	12	13	7	4	504	2.5	1	R	Ax	1	≥2
R. fulgida	12	1.5	19	7	8	4	5	98	0.75	0	R	Ax	1	<2
R. galeottii	13	1.5	7	6	5	4.5	0	8	1	0	P	Ax	0	<2
R. geminiflora	11	1.5	12.5	7.5	7	8.5	0	1	1	0	P	Ax	0	<2
R. gracilis	4.5	1.5	14	8	5	3	0	54	0.25	0	R	Ax	1	<2
R. grantii	19.5	2.5	16.5	8	7	7	1	85	1.5	0	W	Te	1	<2
R. haenkeana	60	10	20	13	16	8	10	46	2	1	R	Ax	1	≥3
R. hapalotricha	20	3	23	18	17.5	21	0	0	NA	0	P	Ax	0	<2
R. harveyana	16	1.5	18	12	10	10	0	0	NA	0	P	Ax	0	<2
R. hookeriana	29	1.5	21	13	14	13	0	0	NA	0	P	Ax	0	<2
R. humboldtiana	13	1	30	10	12	11	10	98	1.5	1	R	Te	1	≥3
R. humilis	17	2	13	14	8	10	0	0	NA	0	P	Ax	0	<2
R. hygrophila	15	1.5	20	10	11	7.5	0	121	0.5	0	P	Ax	1	<2
R. incomta	13	3	27	15	11	11	0	58	0.5	0	P	Ax	0	<2
R. inflata	28.5	1.5	32	13.5	10	7	13.5	46	0.75	1	R	Ax	1	CL
R. insignis	–	–	–	–	–	–	–	–	–	–	P	Ax	–	<2
R. inundata	12	2	15	5	3.5	3	1	20	0.5	1	R	Ax	0	<2
R. ischnopoda	7	2	23	10	14	8	5	123	2	1	R	Ax	1	<2
R. jaliscana	24	4	23	15	10	10	5	57	2.5	0	Y	Te	1	≥2
R. jimulensis	10	3	17	11	10	11	0	113	1	0	P	Ax	1	<2
R. jussieuoides	36	1.5	10	5	21	15	0	19	0.75	1	P	Ax	0	<2
R. lactea	10	2	12	10	5	5	0	18	1	0	P	Ax	0	<2
R. leucantha	23	2	18	11	10	7.5	0	0	NA	0	W	Ax	0	<2
R. longifilamentosa	20	2	3	3	6	7.5	0	56	2	–	Y	Te	1	CL
R. longipedunculata	15	2	16	5.5	3	3	2	172	1	0	R	Ax	1	≥2
R. longepetiolata	–	–	–	–	–	–	0	–	–	0	P	Ax	0	<2
R. macrophylla	10	1.5	35	9	13	5	7	165	0.75	1	R	Ax	1	≥2
R. macrosolen	39	3	25	20	15	15	0	115	1.5	0	W	Te	1	<2
R. magniflora	11	2	22	10	14	20	0	1	0.75	0	P	Ax	0	<2
R. malaca	12.5	5	23	19	17	13	22	316	4	1	Y	Te	1	CL
R. matagalpae	29	2	7	8	10	7.5	0	1.5	0.5	0	P	Ax	0	<2
R. matudae	12	2	20	7	7	6	1	114	1.5	1	R	Ax	1	≥2
R. maya	46	2.5	20	11	20	16	0	0	NA	0	P	Ax	0	<2

Continued.

Appendix 2. Continued.

	TL	TW	ThL	ThW	LL	LW	SE	PL	PT	Lo	Co	If	Pr	Ht
R. mcvaughii	20	2.5	40	7	20	12	10	4	1	1	R	Ax	0	≥2
R. megachlamys	135	3	8	5	18	18	10	30	1	1	W	Ax	1	CL
R. menthifolia	31	2.5	25	16	18	15	0	1	0.75	0	P	Te	0	<2
R. menthoides	9	1	9.5	6	5	3	0	0	NA	0	P	Ax	0	<2
R. metallica	12	1	12.5	6	7	7	0	33	1.5	0	P	Te	0	<2
R. metzae	32	3	21	9	13	12	0	118	1.5	0	W	Ax	1	<2
R. morongii	13	2	20	12	11	11	0	0	NA	0	W	Ax	0	<2
R. multifolia	28	2	25	15.5	14	20	0	0	NA	0	P	Ax	0	<2
R. nitida	22	2	12	10	10	8	0	110	1	0	P	Ax	1	<2
R. nobilis	95	2.5	20	10	25	25	15	3	0.75	0	W	Ax	0	<2
R. novogaliciana	20	2	35	8	12	5.5	6	11	0.75	1	R	Ax	0	≥2
R. nudiflora	9	2	16	9	10	8	0	83	0.5	0	P	Ax	0	<2
R. oaxacana	15	1.5	14	7.5	8	7.5	0	0	NA	0	P	Ax	0	<2
R. paniculata	12	1	7	4	4	4	1	131	1	0	P	Ax	0	<2
R. pearcei	4	2	36	13	5	4	4	238	1	0	R	Ax	1	<2
R. pedunculosa	12	2	13	6	5	5	0	150	0.75	0	P	Ax	1	<2
R. pennellii	14	1.5	11	9	10	7	0	300	2.5	0	W	Ax	0	<2
R. petiolaris	20	3	44	30	23	16	30	23	5	1	Y	Te	0	≥3
R. pilosa	23	2	20	9	17.5	16	0	6	0.5	0	P	Ax	0	<2
R. pittieri	10	1.5	5	4	5	4	0	170	1	0	P	Ax	0	<2
R. pringlei	30	1.5	24	13	11	17	0	20	1.5	0	W	Te	0	<2
R. proxima	21	3	17	11	10	8	0	385	2.5	0	W	Te	1	<2
R. puri	27	2	11.5	3.5	16	17	0	2	1	0	P	Ax	0	<2
R. purshiana	13	2	15	10	10	10	0	1	1	0	P	Ax	0	<2
R. pygmaea	9	–	–	5	3.5	3.5	0	0	NA	0	P	Ax	0	<2
R. riopalenquensis	16	1.5	12	8	9	7	0	20	1	0	P	Ax	0	<2
R. rubra	14	1	10	5	4	3.5	0	139	1	0	Y	Ax	0	<2
R. ruiziana	7	2	25	13	5	8	2	164	1.5	0	R	Ax	0	<2
R. runyonii	11	1.5	17	12	11	11	0	112	1	0	P	Ax	1	<2
R. salviaefolius	8	6	18	7	9	6	11	189	2	1	R	Te	1	<2
R. sanguinea	15	1.5	12	5	3	3	1	65	0.75	0	R	Ax	0	<2
R. saulensis	15	2	17	6	7	5	0	155	0.75	0	W	Ax	0	<2
R. speciosa	28	4	17	16	16	15	6	14	1	1	Y	Ax	0	≥2
R. standleyi	2.5	2	9	5	3	2.5	0	156	1.5	0	Y	Te	1	≥2
R. stemonacanthoides	12	1.5	12.5	4	5	4.5	0	176	1	0	P	Ax	0	<2
R. strepens	19	2.5	13	10	14	16	0	5	0.75	0	P	Ax	0	<2
R. steyermarkii	8	5	15	14.5	10	10	12	35	1.25	1	Y	Ax	0	CL
R. subsessilis	15	2	24	5	11	8	1	246	1.25	1	R	Ax	1	<2
R. tarapotana	25	2	15	6.5	15	13	0	0	NA	0	P	Ax	0	<2
R. terminale	8.5	1	9.5	5.5	4	4	0	0	NA	0	W	Ax	0	<2
R. tomentosa	14	3	22	8	15	6.5	10	18	1.25	1	R	Ax	0	<2
R. tuberosa	7	2	17	6	13	16	0	81	1	0	P	Ax	0	<2
R. tubiflora	30	3	38	17	16	12	0	25	2	0	W	Ax	0	≥2
R. tuxtlensis	15	2	20	7	5	5	0	70	2.5	1	Y	Te	1	<2
R. verbasciformis	6	8	33	25	13	12	21	145	3.5	0	Y	Te	1	<2
R. villosa	9	1.5	31	7	4	4.5	1	3	0.5	0	R	Ax	0	<2
R. yurimaguensis	24	2.5	17.5	10	12.5	9	0	0	NA	0	P	Ax	0	<2

Supplementary Material

The following supplementary material is available for this article:

Figure S1. 154-taxon Bayesian Majority Rule tree showing ancestral reconstructions of corolla color. Details of tree and reconstructions follow Figure 2.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1558-5646.2008.00398.x
(This link will take you to the article abstract).