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Are spurred cyathia a key innovation? Molecular systematics and trait evolution in the slipper spurges (Pedilanthus clade: *Euphorbia*, Euphorbiaceae)[1]

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The study of traits that play a key role in promoting diversification is central to evolutionary biology. Floral nectar spurs are among the few plant traits that correlate with an enhanced rate of diversification, supporting the claim that they are key innovations. Slight changes in spur morphology could confer some degree of premating isolation, explaining why clades with spurs tend to include more species than their spurless close relatives. We explored whether the cyathial nectar spur of the Pedilanthus clade (*Euphorbia*) may also function as a key innovation. We estimated the phylogeny of the Pedilanthus clade using one plastid (*matK*) and three nuclear regions (ITS and two *G3pdh* loci) and used our results and a Yule model of diversification to test the hypothesis that the cyathial spur correlates with an increased diversification rate. We found a lack of statistical support for the key innovation hypothesis unless specific assumptions regarding the phylogeny apply. However, the young age (hence small size) of the group may limit our ability to detect a significant increase in diversification rate. Additionally, our results confirm previous species designations, indicate higher homoplasy in cyathial than in vegetative features, and suggest a possible Central American origin of the group.

Key words: cyathium; diversification rate; *Euphorbia*; Euphorbiaceae; *G3pdh*; key innovation; *matK*; nectar spur; Pedilanthus; phylogeny.

Understanding the factors that promote disparities in the rate of diversification among lineages is central to evolutionary biology. The concept of key innovation was used by Simpson (1953) to refer to a trait or group of traits that allow a lineage to occupy a new adaptive zone. Because the occupation of a novel adaptive zone tends to promote diversification and the accumulation of more species, the term key innovation has come to refer to traits that contribute to an increase in the intrinsic species diversification rate of a taxon (Hunter, 1998; Galis, 2001; Ree, 2005; Kay et al., 2006). Under this revised definition, the hypothesis that a trait is a key innovation is ideally supported by three kinds of evidence (Hunter, 1998; Galis, 2001, and references therein): (1) The taxon having the trait has a higher rate of diversification than closely related taxa lacking the trait, (2) there is a reasonable ecological or functional model to justify a causal link between the trait and increased diversity, and (3) analogous traits are consistently associated with increased diversification rates. The inherent association between floral traits and reproduction in angiosperms has led evolutionary biologists to focus on these traits as key players in the differential diversification of clades of flowering plants (Kay et al., 2006). In this paper, we explore whether the unusual spurred inflorescences that characterize the Pedilanthus clade of *Euphorbia* L. have played a key role in increasing its diversification rate compared to close relatives.

Among the few plant traits that have been studied carefully and have been shown to meet all three criteria for key innovations are floral nectar spurs (Hodges, 1997; Ree, 2005; Kay et al., 2006). Floral spurs are formally defined as hollow, slender, sac-like appendages of a perianth organ, typically containing nectar (Harris and Harris, 2001; Neilson et al., 1950). The floral spurs of *Aquilegia* L. are a thoroughly studied example of this morphological trait. It has been shown that the rate of diversification is higher in *Aquilegia* than in closely related, spurless Ranunculaceae (Hodges and Arnold, 1995). Additionally, a plausible causal model of how nectar spurs could promote increased diversification has been proposed: nectar spurs allow for the distance between the floral reward and the reproductive organs to evolve rapidly without concomitant changes in the floral reproductive organs themselves, thereby increasing the rate at which lineages develop premating isolation (Hodges, 1997). It has been suggested that nectar spurs could promote reproductive isolation, and therefore the rate of speciation,

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through either specialization on different pollinators or differential placement of pollen on the same pollinators. In support of this inference, there is a statistically significant correlation between floral spurs and increased species number in multiple spurred/spurless sister clades (Hodges, 1997; Kay et al., 2006). Sixteen independent origins of nectar spurs have been documented so far, 12 of which are associated with clades that are more species rich than their sister clades (Kay et al., 2006).

Until now, studies have focused primarily on florally derived nectar spurs. However, floral organs are not unique in their ability to produce nectar, and in some taxa, extrafloral nectaries have been shown to play a role in pollination (e.g., in *Euphorbia* L., *Acacia* L., and Marcgraviaceae). Of the extrafloral nectaries that function in pollination, to our knowledge, only one clade produces an extrafloral nectar spur: the Pedilanthus clade of *Euphorbia*. Like all *Euphorbia*, the Pedilanthus clade has reduced flowers organized in a specialized pseudanthial inflorescence, the cyathium. Members of the Pedilanthus clade are unusual in having a strongly zygomorphic cyathium with a nectar-containing spur that is derived from the fusion of petaloid appendages of nectar glands associated with an inflorescence involucre (Fig. 1). To reach the nectar reward, pollinators (primarily hummingbirds; N. I. Cacho, personal observation; Dressler, 1957) probe the cyathial spurs and in so doing contact the staminate or pistillate flowers. Thus, these cyathial spurs in the Pedilanthus clade have an analogous function to floral nectar spurs, but develop at a distinct level of organization, the inflorescence rather than the flower. Therefore, they present a unique opportunity to determine whether the pattern of increased diversity in clades associated with nectar spurs is limited to floral spurs or could extend to all nectar spurs that function in plant–pollinator interactions.

The 15 species that comprise the Pedilanthus clade are characterized by zygomorphic cyathia that usually resemble slippers, as reflected in their many common names (slipper spurges, zapatitos, and queen's slipper) and also account for the scientific name of the genus to which these species were traditionally assigned: *Pedilanthus* Necker ex Poit. ("foot flower"). However, molecular phylogenetic research has shown that the slipper spurges form a clade that is embedded within *Euphorbia* sensu lato (Steinmann and Porter, 2002). Based on this result, all species names have been transferred from *Pedilanthus* to *Euphorbia* (Steinmann, 2003).

The Pedilanthus clade exhibits great morphological and ecological diversity for a group of its size (Fig. 2). Habit ranges from succulent leafless shrubs about a meter in height [e.g., *E. cymbifera* (Schltdl.) V.W.Steinm.], to evergreen treelets a few meters tall [e.g., *E. finkii* (Boiss.) V.W.Steinm., *E. peritropoides* (Millsp.) V.W.Steinm.], to deciduous trees up to 8 m tall [e.g., *E. coalcomanensis* (Croizat) V.W.Steinm.]. Slipper spurges occur in diverse habitats, including mesic tropical forests, dry deciduous forests, and true deserts such as the Sonoran Desert or the Tehuacán Desert in central Mexico. Leaf size, shape, persistence, and indumentum are all variable. Some species produce tuberous roots, adventitious root buds, or rhizomatous stems, whereas other species lack any obvious adaptations for vegetative reproduction or perennation (Dressler, 1957; N. I. Cacho, personal observations). There is abundant variation in cyathium size and color pattern, spur elongation and coloration, and cyathium bract morphology and phenology (Fig. 3). This variation may correlate with different pollination systems to some extent. Most species of the group are thought to be hummingbird-pollinated (Dressler, 1957), but *E. diazlunana* (J.Lomelí & Sahagún) V.W.Steinm. has been reported to be pollinated by hymenopterans (Sahagún-Godínez and Lomelí-Sención, 1997) and, based on its morphology, *E. tehuacana* (Brandegee) V.W.Steinm. is also likely to be insect-pollinated, although formal pollination studies in the group are lacking.

Twelve of the fifteen species in the Pedilanthus clade are restricted to Mexico. The Mexican species vary in their geographical range from *Euphorbia lomelii* V.W.Steinm., estimated to occupy some 300 000 km^2 in the deserts around the Gulf of California, to the microendemic *E. conzattii* V.W.Steinm. with a range of 0.2 km^2 on a single mountaintop (Olson et al., 2005). Of the three species with distributions extending beyond Mexico's borders, the southernmost populations of *E. calcarata* (Schltdl.) V.W.Steinm. occur in northern Guatemala, whereas *E. personata* (Croizat) V.W.Steinm. has disjunct populations as far south as Costa Rica. Finally, *E. tithymaloides* L., by far the most widespread species of the clade, has a range that includes Mexico, Florida, northern South America, Central America, and most islands in the Caribbean. In addition to an unusually broad distribution, *E. tithymaloides* is also notable in the group for the degree of infraspecific differentiation, with eight subspecies recognized (Dressler, 1957).

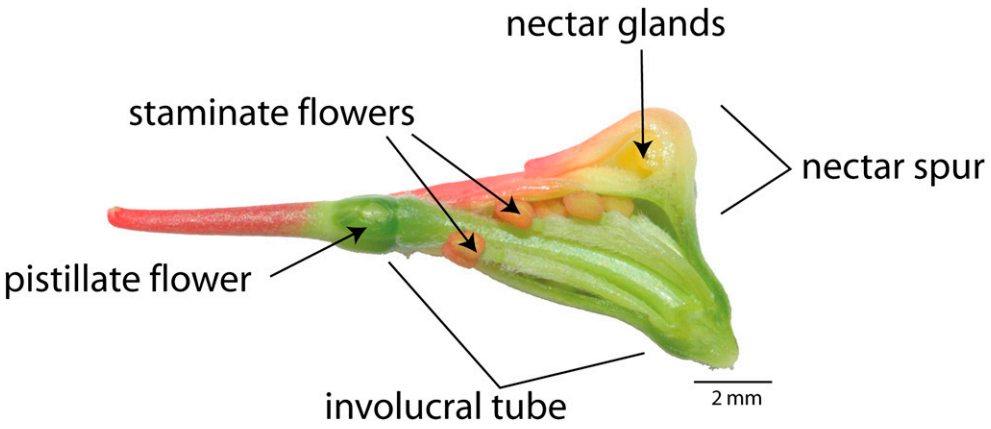

Fig. 1. Longitudinal midsection of the spurred zygomorphic cyathium of *Euphorbia tithymaloides* subsp. *padifolia.* The terminal pistillate flower, single staminate flowers, involucral tube, nectar glands, and spur concealing nectar glands are indicated.

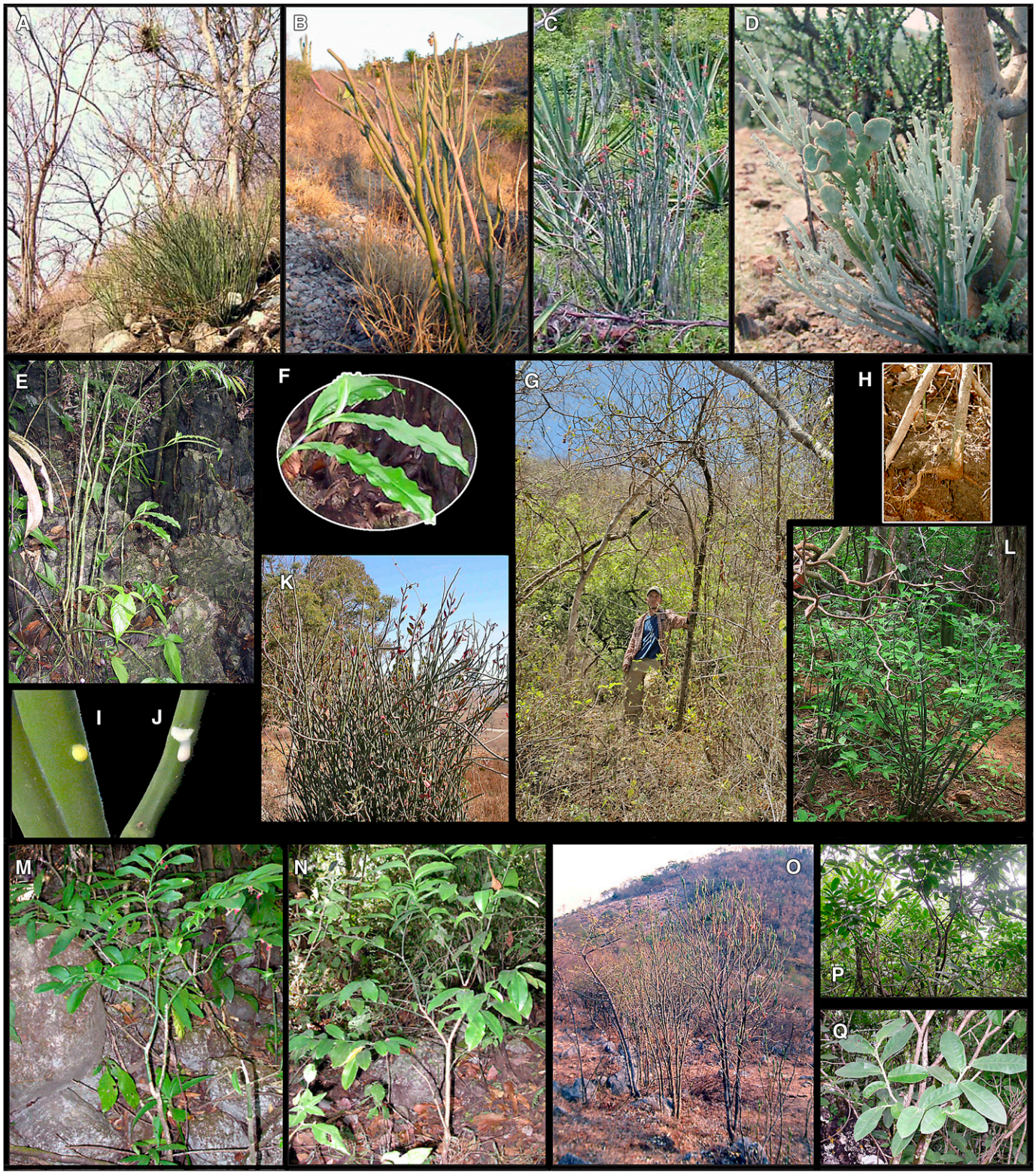

Fig. 2. Diversity in habit in the *Pedilanthus* clade. (A) *Euphorbia diazlunana*. (B) *E. tehuacana*. (C) *E. personata*. (D) *E. lomelii*. (E) *E. finkii*. (F) Glossy leaves of *E. finkii*. (G) *E. calcarata*. (H) Root of *E. calcarata*. (I) Yellow latex of *E. diazlunana*. (J) White latex of *E. peritropoides*. (K) *E. cyri*. (L) *E. tithymaloides*. (M) *E. peritropoides*. (N) *E. conzattii*. (O) *E. coalcomanensis* during the dry season. (P) Canopy of *E. coalcomanensis* during rainy season. (Q) Leaves of *E. coalcomanensis*.

Fig. 3. Diversity in reproductive morphology in the *Pedilanthus* clade and some close relatives. (A) *E. bracteata*, in cultivation. (B) *E. bracteata* in the wild; note bract and fruit color. (C) *E. personata*, inflorescence cluster. (D) *E. personata*, fruit. (E) *E. diazlunana*. (F) *E. diazlunana*, in cultivation. (G) *E. tithymaloides* subsp. *tithymaloides* fruit. (H) *E. tithymaloides* subsp. *padifolia*. (I) *E. lomelii*, image by T. B. Kinsey (http://www.fireflyforest.net/firefly/), reproduced with the author's permission. (J) Persistent, dry inflorescence of *E. cyri*; note large and persistent bracts. (K) *E. calcarata*. (L) *E. finkii* from herbarium material. (M) *E. colligata*. (N) *E. cymbifera*. (O) *E. conzattii*. (P) *E. peritropoides*; note peduncles. (Q) Persistent, dry inflorescence of *E. coalcomanensis*. (R) *E. coalcomanensis*; note bright bracts. (S) Gland of *E. umbelliformis*, image by P. E. Berry. (T) Cyathia of *E. umbelliformis*, image by P. E. Berry (U) *E. gollmeriana*. (V) *E. pteroneura*. (W) *E. leucocephala*.

Prior phylogenetic hypotheses for the Pedilanthus clade are based exclusively on morphological characters (Dressler, 1957) or considered only a few species (Steinmann and Porter, 2002); and both are poorly resolved. In recent years, new species have been described (Dressler and Sacamano, 1992; Sahagún-Godínez and Lomelí-Sención, 1997), and extant populations of several others have been located (Lomelí-Sención and Sahagún-Godínez, 2002; Olson et al., 2005), giving us the opportunity to conduct a comprehensive phylogenetic study in this group.

In this paper, we provide the first detailed molecular phylogenetic analysis of the Pedilanthus clade and use the results to test the hypothesis that the cyathial spur, like the floral spur of *Aquilegia*, is a key innovation. We also use our phylogenetic results to study trait evolution in this small yet morphologically and ecologically diverse group, to assess the exclusivity of some traditionally named species, to test Dressler's (1957) hypothesized species groups, and to test the hypothesized Mexican origin of the group (Dressler, 1957).

MATERIALS AND METHODS

Taxon sampling—Plant material was collected in the wild for 14 of the 15 described species of the Pedilanthus clade. We were unable to locate *Euphorbia dressleri* V.W.Steinm. despite intensive fieldwork in and around the sole locality from which it has been reported. To our knowledge, there are no cultivated individuals of this species. We therefore think that this species has likely become extinct. For the remaining species of the Pedilanthus clade, representatives of multiple populations per species (up to six) were included when possible (see Appendix 1 for taxa included in this study).

We sequenced selected outgroups to represent three of the four major clades of *Euphorbia*, as defined by Steinmann and Porter (2002): clade B (*E. esula* L., *E. cyparissias* L.), clade C [*E. umbelliformis* (Urb. & Ekman) V.W.Steinm. & P.E.Berry, *Euphorbia pteroneura* A.Berger, *E. gollmeriana* Klotzsch ex Boiss., *E. milii* Des Moul., *Euphorbia umbellata* (Pax) Bruyns], and clade D [*E. leucocephala* Lotsy, *E. heterophylla* L., *E. oerstediana* (Klotzsch & Garcke) Boiss.]. Representatives of eight outgroups were collected in the field, two (*E. milii, Manihot esculenta* Crantz) were collected from cultivation, and sequences for other *Euphorbia* outgroup taxa were downloaded from GenBank (see Appendix 1 for detailed information).

DNA extraction, PCR amplification, and sequencing—Genomic DNA was extracted from plant tissue dried in silica gel (Chase and Hillis, 1991) using either the CTAB method as outlined by Doyle and Doyle (1987) or using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). The internal transcribed spacer region (ITS), consisting of ITS1 and ITS2 and the 5.8S ribosomal nuclear gene, was amplified with primers ITS1 and ITS4 (White el al., 1990; Appendix S1, see Supplemental Data with the online version of this article), and sequenced with the ITS1, ITS2, ITS3, and ITS4 primers. About 1500 bp of the *matK* sequence were amplified with primers trnK3914F (Johnson and Soltis, 1994) and p6R (Nyffeler et al., 2005). Due to the presence of extremely AT-rich regions, six additional newly designed sequencing primers were used (Appendix S1, see online Supplemental Data). The *glyceraldehyde 3-phosphate dehydrogenase-subunit C* (*G3pdhC*) gene was initially amplified with the primers GPDX7F and GPDX9F (Strand et al., 1997), gel purified with QIAquick Gel Extraction Kit (Qiagen, Valencia, California, USA), ligated into a pGEM T-Vector (Promega, Madison, Wisconsin, USA), cloned in *E. coli* DHB-5α competent cells (Invitrogen, Carlsbad, California, USA), reamplified, and sequenced. Two loci were separated by this cloning procedure, here referred to as *G3pdhC-A* and *G3pdhC-B*. Locus-specific primers were designed and used for amplification, cloning when necessary (eight clones per accession, same procedure as already outlined), and sequencing (AF1/AR1 and BF1/BR1 in online Appendix S1).

Most PCR reactions included 2.5 μL of M891A PCR-Buffer (Promega), 2.5 μL of 25mM MgCl2, 0.5 μL dNTP mix (2.5 mM each), 0.5 μL of each primer (10 μM), and 0.625 units of Flexi-Taq (Promega) in a 25-μL reaction. The amplification of *matK* required the use of buffer M890A (Promega) and BSA (0.8%). For ITS and *G3pdhC-A/B*, the cycling conditions consisted of an initial denaturation at 94°C for 10 min, followed by a three-cycle touchdown decreasing 2° per cycle (94°C for 30 s, 58°/56°/54°C for 60 s, 72°C for 90 s); 31 additional cycles of 94°C for 30 s, 54°C for 60 s, 72°C for 90 s; and, a final extension of 7 min at 72°C. The cycling conditions for *matK* were initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 50 s, 52°C for 70 s, 72°C for 90 s; and a final extension of 5 min at 72°C. PCR products were cleaned and diluted using Ampure Magnetic Beads (Agencourt Biosciences, Beverly, Massachusetts, USA) following the manufacturer's protocol.

Sequencing reactions consisted of 0.5 μL of BigDye Terminator v. 3.1 mix (Applied Biosystems), 2.0 μL of 5× dilution buffer (Applied Biosystems), 5 pmol of primer, DMSO (10%), and ~0.2 μg of template DNA in a final reaction volume of 10 μL. Cycle conditions consisted of an initial denaturation at 95°C for 3 min; 50 cycles of 96°C for 10 s, 58°C for 4 min; and a final extension of 7 min at 72°C. Excess dye terminators were removed using the CleanSeq magnetic bead sequencing reaction clean up kit (Agencourt Biosciences). Samples were electrophoresed on an Applied Biosystems 3730xl automated DNA sequencing instrument, using 50-cm capillary arrays and POP-7 polymer, at the University of Wisconsin-Madison Sequencing facility.

Sequences were assembled and edited in the program Sequencher v. 4.7 (Gene Codes Corp., Ann Arbor, Michigan, USA), and manually aligned in the program MaClade v. 4.05 (Maddison and Maddison, 2002). In general, alignments were unambiguous, at least within the ingroup. Where alternative alignments that invoked a similar number of indel and substitution events could be identified, we selected the one that minimized the number of parsimony informative characters that were generated. For both loci of *G3pdhC*, a minimum of eight clones per individual was examined. When more than one allele was recovered from an individual, from five to eight additional clones were sequenced. PCR error and PCR recombination were assessed by manual examination of the sequences; potential PCR recombinants were excluded from the analyses.

Phylogenetic analyses—We examined our data sets for phylogenetic signal using g1 statistics and permutation tail probability tests (PTP) as implemented in the program PAUP* v. 4.0b10 (Swofford, 2002). Data sets were then analyzed individually and in combination. Only accessions with data for three or more partitions were included in combined analyses. Congruence among data partitions was explored in a parsimony framework using the incongruence length difference (ILD) test (Farris et al., 1994) as implemented in PAUP*. Sources of conflict were identified by deletion of potentially conflicting taxa (based on examination of individual gene topologies). For ILD analyses, 10 000 replicates of flat-weighted parsimony heuristic searches were conducted, with 10 random additions, holding 10 trees per step, tree-bisection-reconnection (TBR) branch swapping, and saving no more than one tree per replicate. Data sets that showed no evidence of conflict were concatenated for combined analysis.

As many as six accessions per species were included in both individual and combined data sets to assess species monophyly. We conducted a set of combined analysis that retained all individuals, which we will refer to as "combined-all". There were only three cases in which distinct alleles were recovered from a single, presumably heterozygous, individual (*E. diazlunana*, *E. calcarata*_01, and *E. colligata* V.W.Steinm._02 for *G3pdhC-A*). We also performed a final combined analysis with a single accession per species ("combined-one"). For this combined analysis, we decided which alleles to use by conducting preliminary parsimony searches with all possible combinations of alleles and selected the set of alleles that yielded the shortest trees.

Maximum parsimony (MP)—For both separate and combined data sets, flat-weighted MP heuristic searches were performed in PAUP* v. 4.0b10 (Swofford, 2002). Starting trees were obtained by 10 000 random addition replicates holding 10 trees per step and keeping best trees only. Searches used the TBR branching swapping algorithm and saved only one tree per replicate. A second search was run to completion starting from the set of most-parsimonious trees and swapping (TBR). Clade support was assessed by 10 000 bootstrap replicates as implemented in PAUP* with the following search settings: 10 random addition replicates, hold = 1, keep = best, TBR, not more than one tree saved per replicate.

Model selection—An appropriate and not overly complex model of molecular evolution was selected under a decision theory framework as implemented in the program DT-ModSel (Minin et al., 2003). We modified the code to evaluate alternative models on a most parsimonious tree rather than a neighbor-joining tree. We only considered models that account for site-to-site rate heterogeneity using a discrete approximation to a gamma distribution of rates (Gamma) rather than those that also allow for a fixed proportion of invariant sites (P-invar), as recommended by Stamatakis et al. (2008; RAxML v. 704 manual).

TABLE 1. Data sets analyzed, including matrices' dimensions, models implemented, and trees obtained. Character status is in reference to the aligned matrices (Cte = constant; PIC = parsimony informative characters; NPIC = nonparsimony informative characters). Number of most parsimonious trees (MPTs), their length (L), consistency (CI), retention (RI), and rescaled consistency (RC) indices are provided for the maximum parsimony (MP) analyses. For the maximum likelihood (ML) analyses, the model, program used, and optimal likelihood score are provided. For ML analyses with RAxML, the numbers in parenthesis for the combined analyses refer to the number of partitions considered. The likelihood score provided for Bayesian analysis corresponds to the likelihood of best state for "cold" chain of run 1; sf = average split frequencies.

Data set	Generals	MP	ML (Garli)	ML (RAxML)	MrBayes
G3pdhC-A	no. taxa = 41	no. MPTs = 9079	HKY+G	GTR+G	HKY+G
Outgroup: *E. cyparissias*	no. chars = 966	L = 1035	–Ln L = 5831.5463	–Ln L = 5830.689202	–Ln L = 5861.54
	cte = 416	CI = 0.775			sf = 0.006415
	NPIC = 176	RI = 0.816			
	PIC = 374	RC = 0.632			
G3pdhC-B	no. taxa = 35	no. MPTs = 156	HKY+G	GTR+G	HKY+G
Outgroup: *E. cyparissias*	no. chars = 798	L = 767	–Ln L = 4111.9547	–Ln L = 4111.678	–Ln L= 4140.33
	cte = 332	CI = 0.850			sf = 0.006610
	NPIC = 146	RI = 0.897			
	PIC = 320	RC = 0.762			
ITS	no. taxa = 64	no. MPTs = 5302	GTR+G	GTR+G	GTR+G
Outgroup: *M. esculenta*	no. chars = 782	L = 1448	–Ln L = 7532.9602	–Ln L = 7535.598	–Ln L = 7597.88
	cte = 378	CI = 0.489			sf = 0.005813
	NPIC = 69	RI = 0.756			
	PIC = 335	RC = 0.370			
matK	no. taxa = 40	no. MPTs = 125	GTR+G	GTR+G	GTR+G
Outgroup: *M. esculenta*	no. chars = 1646	L = 462	–Ln L = 4915.4074	–Ln L = 4916.249	–Ln L = 4948.77
	cte = 1284	CI = 0.877			sf = 0.004931
	NPIC = 205	RI = 0.895			
	PIC = 157	RC = 0.784			
Combined-all	no. taxa = 37	no. MPTs = 84	GTR+G	GTR+G	unlinked
Outgroup: *E. cyparissias*	no. chars = 4161	L = 1799	–Ln L = 14886.503	–Ln L (1 partition) = 14889.380	–Ln L = 14737.85
	cte = 2957	CI = 0.833		–Ln L (4 partitions) = 14238.121	sf = 0.003480
	NPIC = 435	RI = 0.867			
	PIC = 769	RC = 0.723			
Combined-one	no. taxa = 25	no. MPTs = 1	GTR+G	GTR+G	unlinked
Outgroup: *M. esculenta*	no. chars = 4161	L = 2934	–Ln L = 19184.935	–Ln L (1 partition) = 19186.333	–Ln L = 18941.15
	cte = 2550	CI = 0.757		–Ln L (4 partitions) = 18332.947	sf = 0.002337
	NPIC = 639	RI = 0.772			
	PIC = 972	RC = 0.584			

Maximum likelihood (ML)—Searches were performed in the program Garli v. 0.95 (Zwickl, 2006) under the optimal model of evolution for each data set and under the GTR+G model in the program RAxML (Stamatakis et al., 2008; see Table 1). The combined data sets were analyzed as a single partition under the GTR+G model of evolution both in Garli and in RAxML, and as four partitions in RaxML, taking advantage of the "per gene branch optimization" setting that allows parameters to be optimized independently among genes even when analyzed assuming the same model of evolution. Support values were obtained by ML bootstrapping with automatic estimation of replicate number in Garli and RAxML (RAxML calculations were carried out on the CIPRES cluster, at the San Diego Supercomputer Center; Miller et al., 2009). For the combined-one analyses, we tested resolved branches to see whether they were significantly better than a polytomy using a likelihood ratio test (as described by Baum et al., 2004).

Bayesian Markov chain Monte Carlo analysis—Metropolis coupled Markov chain Monte Carlo (MCMCMC) tree sampling (Larget and Simon, 1999; Mau et al., 1999) was implemented in the program MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) under the optimal model of evolution. For the combined data sets, we analyzed each of the four partitions under its best fitting model, linking only the topology and branch lengths across partitions (unlinked parameters: tratio, revmat, statefreq, and shape). Two independent analyses of two runs each were performed, with the following parameters: nchains = 4, ngens = 1 000 000, sampfreq = 100, temp = 0.2 (*G3pdhC-A/B*), 0.04 (ITS), and 0.07 (*matK*). Heat was selected based on a preliminary evaluation of mixing guided by split frequencies and acceptance rates. Based on generation-by-likelihood plots, from 10–15% of the samples were discarded as burn-in.

Topology tests—Support for alternative topologies was explored in the MP framework using Wilcoxon sign-ranked tests (Templeton, 1983) as described by Larson (1994) and implemented in PAUP*. In the MCMC framework, we assessed support for alternative topologies by determining their frequency in the posterior distribution. We tested the following clade relationships suggested by Dressler (1957): (*E. conzattii* + *E. coalcomanensis* + *E. cymbifera*); (*E. lomelii* + *E. bracteata* Jacq. + *E. tehuacana* + *E. cyri* V.W.Steinm.); [*E. peritropoides* (Millsp.) V.W.Steinm. + *E. finkii*], and; (*E. tithymaloides* + *E. personata*). We were not able to test (*E. calcarata* + *E. dressleri*) because *E. dressleri* was not available.

Trait evolution—Thirty-four discrete morphological characters were scored based on field observations, herbarium specimens, and the literature. Character states and scoring are presented in Appendices S2 and S3 (see online Supplemental Data).

The 34 characters were mapped onto the ML phylogeny derived from the combined-one data set (one accession per species). We used both MP and ML approaches to map characters, as implemented in the program Mesquite v. 2.01 (Maddison and Maddison, 2009). For ML, a one-parameter Markov model (MK-1) of character evolution was implemented, then visualized according to the proportional likelihood at each node.

Testing cyathial spurs as key innovations—New methods for studying differential diversification of lineages have been developed in recent years (e.g., Ree, 2005; Maddison et al., 2007). Most of these newer methods are premised on including a representative sample of taxa that have or lack the putative key innovation. The difficulties we faced in obtaining a representative sample

of outgroups, combined with our nearly exhaustive sampling in the ingroup, make these methods invalid for our data set. Instead, we decided to use an older method (Sanderson and Donoghue, 1994) that makes use of a phylogeny and relative age estimates for key nodes, but does not stipulate sampling beyond that needed to establish the number of species per clade.

To estimate a shift in diversification rate associated with cyathial nectar spurs, we used our combined-one ML phylogeny and a "pure birth" approach to modeling diversification as implemented in the program LRDiverse v. 0.8 (M. Sanderson, as described in Sanderson and Donoghue [1994, 1996]). This method uses a three-taxon analysis of two ingroup clades that have the putative key innovation, the spurred zygomorphic cyathium in this case, and an outgroup taxon that lacks the trait. Based on the relative ages of the root and ingroup nodes, and the number of species in each of the three clades, the program evaluates the likelihood of clades of the observed size given one of several nested models of diversification and uses likelihood ratio tests to identify the optimal model.

The sister group to the Pedilanthus clade most likely includes between one and seven species (Steinmann and Porter, 2002; Steinmann et al., 2007; V. W. Steinmann, unpublished data). Guided by the most recent publications on phylogenetics of the New World members of clade C of *Euphorbia*, we conducted the analysis under four alternative species counts for the sister group of the Pedilanthus clade: one species (*E. sinclairiana* Benth. [= *E. elata* Brandegee]), as suggested by the analysis of Steinmann and Porter (2002), two species, four species, or seven species (either the *Euphorbia* section *Cubanthus* or the *E. pteroneura* clades). We also considered the scenario of granting species status to the subspecies of *E. tithymaloides*.

We estimated relative nodal ages for the nodes "Outgroup + Pedilanthus clade" (stem node), and "Pedilanthus subclades" (crown node; see Fig. 4) in our ML phylogeny using the program r8s (Sanderson, 2003) under three different methods: molecular clock (Langley-Fitch; LF), nonparametric rate smoothing (NPRS), and penalized likelihood (PL). For each method, we obtained 95% confidence intervals for each of the two estimated relative nodal ages by recalculating ages on 100 trees of the appropriate topology randomly selected from the Bayesian posterior distribution (Perl script for sampling trees at random from the posterior available upon request). For the PL method, the appropriate value of λ was estimated using a cross validation procedure (15 increments of 0.25, starting at zero) on 10 trees of appropriate topology randomly sampled from the Bayesian posterior distribution following Scherson et al. (2008). We then used both the upper and lower bounds of the nodal ages confidence intervals for analyses of diversification rate in LRDiverse.

LRDiverse assumes that branching events follow a Poisson distribution in any given lineage, governed by a single rate parameter that is assigned to each branch of a three-taxon phylogeny ("pure birth" or Yule model). A likelihood ratio test ($\beta = -2LR$) is used to assess goodness of fit of successively less constrained models relative to the completely unconstrained model under which each branch has its own rate of diversification. The five models compared in LRDiverse are, in order of increasing complexity (see LRDiverse manual; Sanderson and Donoghue, 1994, 1996): a one-parameter model (no shift in diversification rate; model 0); three two-parameter models (models 1–3), only one of which (model 1) is compatible with a key innovation hypothesis, and the unconstrained, three-parameter model (model 4). We analyzed our data under the assumption that the internal branch has the same diversification rate as the one subtending the outgroup (mode 0 in LRDiverse). In all cases, we used 1000 replicates of Monte Carlo simulation to statistically evaluate whether a simpler model is rejected in favor of a more complex model.

Finally, we added Pedilanthus to Hodges' (1997) one-tailed sign test analysis of nectar spurs as key innovations across angiosperms and conducted a sister group comparison as outlined by Slowinski and Guyer (1993) under the different outgroup and species scenarios already outlined.

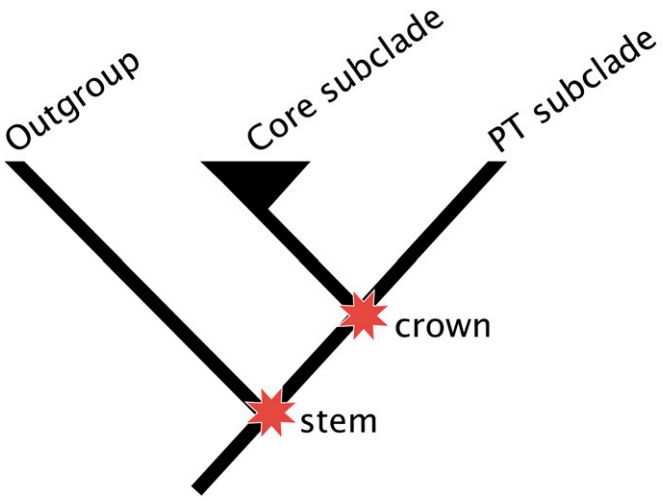

Fig. 4. Three-taxon model used to assess diversification rates in the Pedilanthus clade. The three taxa in the model correspond to: Outgroup (see Results section for the alternative scenarios considered), the "core" Pedilanthus clade (ingroup 1), and the PT subclade of the Pedilanthus clade (ingroup 2). The branches evaluated for a potential shift in diversification rate correspond to the ones subtending these three clades. Relative ages were estimated using r8s for the stem and crown nodes (marked with stars) of the branch subtending the ingroup.

RESULTS

Phylogenetics—Individual data matrices for *G3pdhC-A*, *G3pdhC-B*, ITS, and *matK* consisted of 966, 903, 782, and 1461 characters, respectively, with differing numbers of taxa. The combined-one molecular data set consisted of 4161 characters and 25 taxa, each of which had data for at least three genes. Details on data matrices, diagnostic statistics, model selection, and trees obtained are presented in Table 1. Figures 5 and 6 show the ML trees for each of the four genes analyzed individually. Four main subclades of the Pedilanthus clade were found in multiple single-gene analyses. For ease of communication, these are labeled M (most species live in mesic environments), X (most species live in xeric environments), PT (the two included species are *E. personata* and *E. tithymaloides*), and F (*E. finkii*).

Discordance among molecular markers—An ILD test applied to the four partitions in the combined-all data set rejected the null hypothesis of congruence among datasets ($P < 0.001$). When the four partitions were compared in a pairwise fashion and a Bonferroni correction was used for multiple tests (six comparisons, $\alpha = 0.05/6 = 0.0083$), significant incongruence was only found for the gene pairs: *G3pdhC-A*/ITS and *G3pdhC-A*/*G3pdhC-B*. Incongruence between these data sets appears to be due to three alleles from *Euphorbia calcarata* (*E. calcarata*_01, *E. calcarata*_04, *E. calcarata*_06,) and one allele from *E. colligata* (*E. colligata*_03) when only ingroup taxa were considered, and *E. leucocephala*, *E. oerstediana*, *E. milii*, and *Manihot* when ingroup and outgroup taxa were considered. There is, thus, no evidence of incongruence between the four genes in regards to interspecific relationships within the Pedilanthus clade.

Species monophyly—Substantial infraspecific differentiation was observed in cases where enough sampling was available (i.e., *E. calcarata*, *E. bracteata*, *E. tithymaloides*), but the individual genes often supported species monophyly. For instance, *G3pdhC-B* has enough resolution to resolve *E. calcarata*, *E. peritropoides*, *E. colligata*, and *E. lomelii* as monophyletic entities. However, it fails to resolve species as monophyletic in the PT subclade (Fig. 6B). Conversely, ITS resolves species as monophyletic in the PT clade, but it fails to support monophyly of *E. bracteata* and *E. colligata* (Fig. 6C).

Combined analyses with multiple accessions per species (selecting alleles from heterozygotes that minimize tree length) resulted in good support for monophyly of all species represented by multiple accessions within the core Pedilanthus clade.

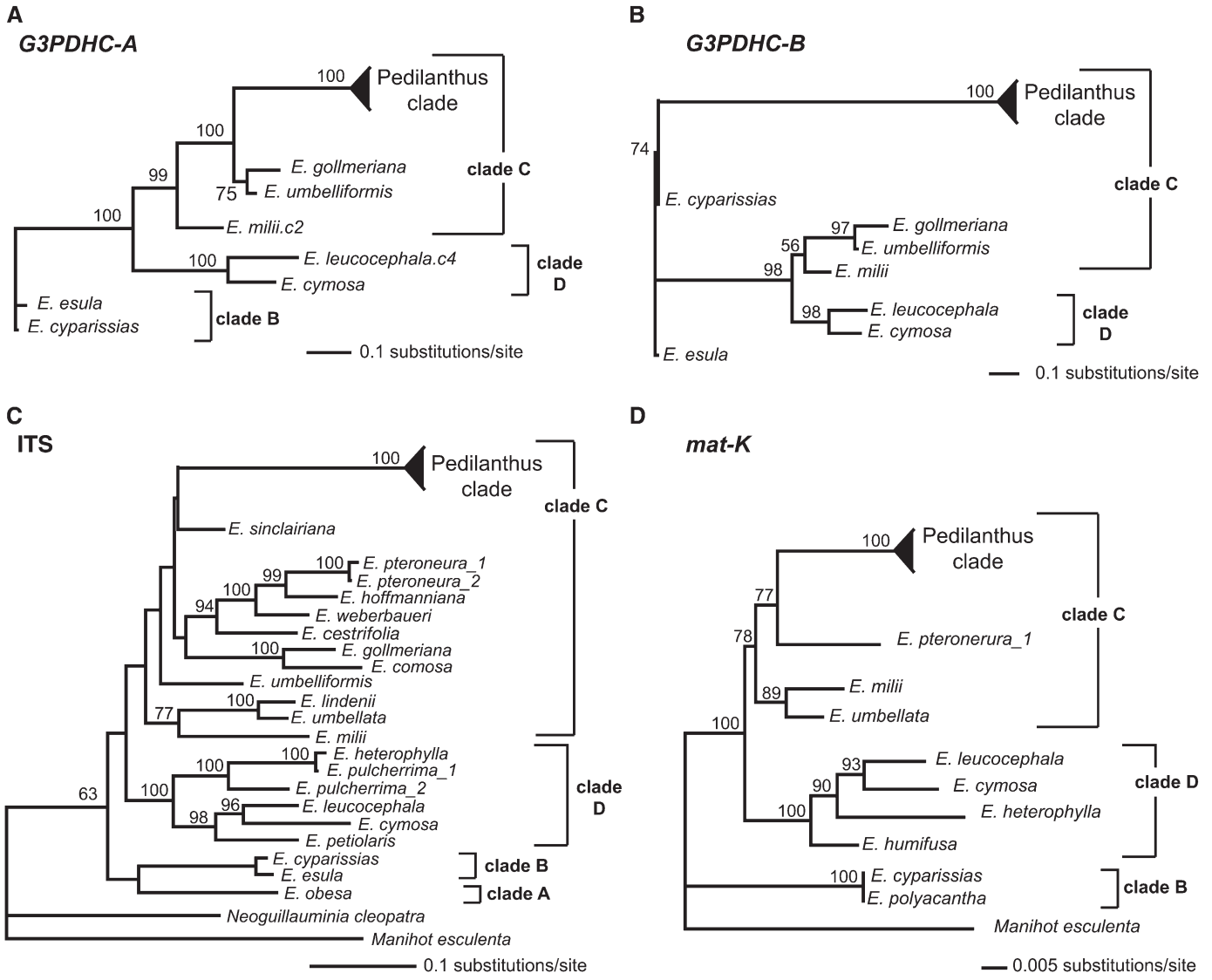

Fig. 5. Maximum likelihood (ML) trees of individual gene analyses showing high support for monophyly of the *Pedilanthus* clade and its relationships to other *Euphorbia* lineages. (A) *G3pdh-A*. (B) *G3pdh-B*. (C) ITS. (D) *matK*. ML bootstrap values ≥50% are shown above branches.

In contrast, reciprocal monophyly of *E. tithymaloides* and *E. personata* is not well supported. Because there is consistent support for monophyly of the PT subclade, ambiguity over reciprocal monophyly of these two species would not substantially influence the results of a combined analysis that uses a single accession per species. The interspecies relationships with the Pedilanthus clade implied by the latter analysis (Fig. 7) are identical to those found when multiple accessions per species are included.

Phylogenetics of the Pedilanthus clade—Monophyly of the Pedilanthus clade was very strongly supported in all analyses and tests. A Templeton test rejected the optimal tree lacking a Pedilanthus clade for the combined-one data ($P < 0.0001$). These results together with the cyathial synapomorphies that unify the group leave no reason to suspect a lack of monophyly of the *Pedilanthus* clade.

While our outgroup sampling is not extensive, our combined and individual gene analyses (Figs. 5, 7) support previous hypotheses (Steinmann and Porter, 2002, Wurdack et al., 2005, Bruyns et al., 2006, Steinmann et al., 2007) that the closest relatives of the Pedilanthus clade are New World members of clade C of *Euphorbia*, here represented by *E. gollmeriana*, *E. pteroneura*, and *E. umbelliformis*. Our sampling is most complete in our ITS analyses. These suggest the single species *E. sinclairiana* as the sister group to the Pedilanthus clade in agreement with Steinmann and Porter (2002), although clade support is lacking for such relationship. The tree estimated from *G3pdhC-B* shows anomalous outgroup relationships—a result that may reflect long-branch attraction in the sparsely sampled outgroup.

Within the Pedilanthus clade, the PT subclade consists of *E. personata* and the *E. tithymaloides* species complex. The PT clade is well supported by the ITS (MLBS = 90; MPBS = 100; PP = 1.0) and *matK* (MLBS = 93; MPBS = 93; PP = 1.0) markers, and is not meaningfully contradicted by either of the *G3pdh* markers. It is also strongly supported by the combined-one (MLBS = 100; MPBS = 99; PP = 1.0) data set, but more weakly supported by the combined-all data set. The shortest trees lacking the PT clade are significantly rejected by the combined-one

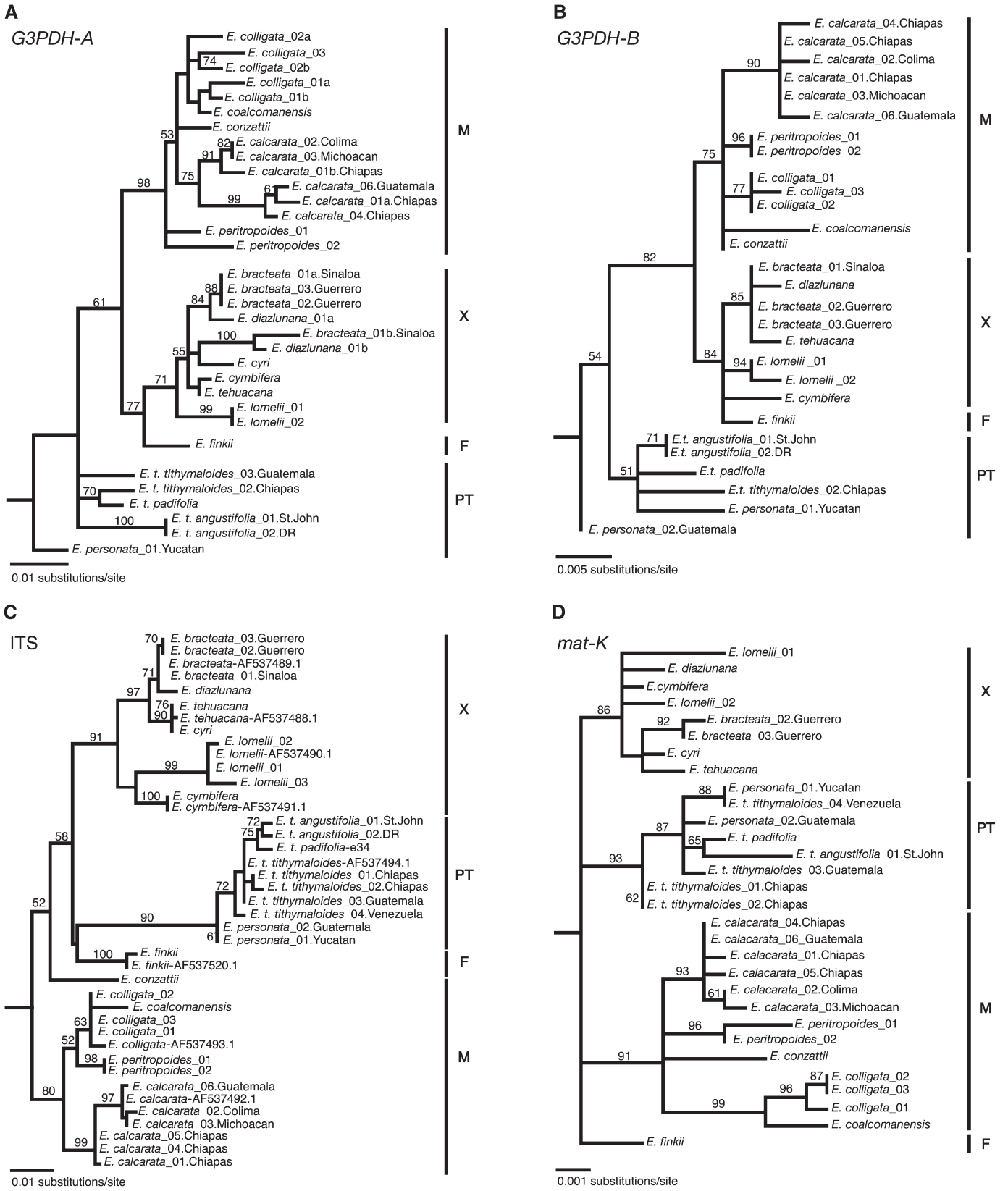

Fig. 6. Maximum likelihood (ML) trees of individual gene analyses showing relationships within the *Pedilanthus* clade. (A) *G3pdh-A*. (B) *G3pdh-B*. (C) ITS. (D) *matK*. ML bootstrap values ≥50% are shown above branches.

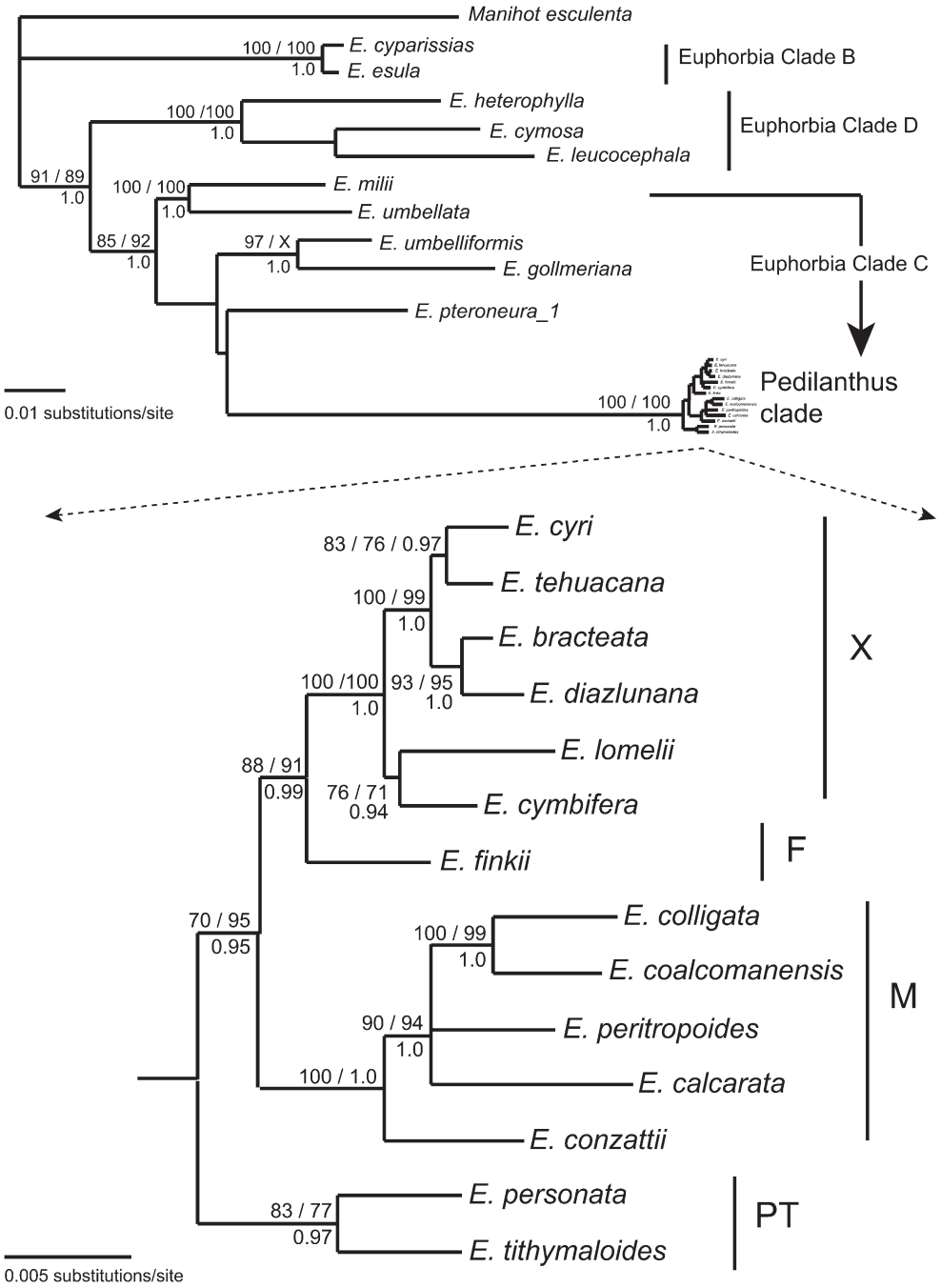

Fig. 7. Maximum likelihood (ML) tree of the combined-one data set. The placement of the Pedilanthus clade (top) and the relationships within it (bottom) are illustrated. Support values ≥50% are as follows: ML bootstrap, MP bootstrap, Bayesian posterior probability.

dataset (P = 0.0158) but not by the combined-all data set (P = 0.3458). The PT subclade was the only group proposed by Dressler (1957) that gained support from our data. Indeed, the other three clades proposed by Dressler (1957) were rejected based on a Templeton test: *E. conzattii* + *E. coalcomanensis* + *E. cymbifera* (P < 0.0001); *E. lomelii* + *E. bracteata* + *E. tehuacana* + *E. cyri* (P < 0.0027), and; E. *peritropoides* + *E. finkii* (P < 0.0001).

With the exception of *E. finkii*, an evergreen species of mesic forests and sole member of subclade F, all five species that occur in mesic forests occur in clade M. Most of these species present a tree habit and large glossy leaves. This contrasts with the xeric clade, X, which includes succulent shrubs of tropical deciduous forest, scrub or desert. Monophyly of the X and M subclades of the Pedilanthus clade is strongly supported by all analyses of individual genes and combined molecular data sets (Figs. 6, 7). Indeed, Templeton tests show that trees lacking either the M or X clades are significantly rejected by the combined-one data (P = 0.0158 and 0.0005, respectively).

G3pdhC-A and *G3pdhC-B* provide moderate support for subclades F and X as sister clades and for these together as sister to

subclade M. Neither *matK* nor ITS conflict with this topology. It is, therefore not surprising that the combined-one data set supports both the (F, X) clade (MLBS = 88; MPBS = 91; PP = 0.99), and the (M (F, X)) clade (MLBS = 70; MPBS = 95; PP = 0.95). However, a Templeton test, conducted with either combined data set, does not allow one to reject alternative relationships among the four subclades.

Trait evolution—Scoring of morphological characters and the resulting morphological matrix are presented in online Appendices S2 and S3. Figure 8 illustrates ancestral state reconstruction (using ML) of six selected traits for the *Pedilanthus* clade. Reconstruction of habit evolution (Fig. 8A) suggests a single origin of the tree habit with one reversal in subclade M. There seems to be a strong correlation between the tree habit and seasonally mesic environments (Fig. 8B). Two wood anatomy traits that might be expected to influence performance in dry vs. wet habitats, vessel-element grouping (Fig. 8C) and intervessel pitting (Fig. 8D), also seem to show some correlation with habitat. Yellow latex was found to be a consistent synapomorphy of a subset of subclade X (Fig. 8E). Spur projection (Fig. 8F) was inferred to show homoplasy, as were spur coloration, cyathium color patterning, the shape of the spur apex, and the spur to involucral tube ratio (data not shown).

Testing key innovation hypotheses—To test the hypothesis that the spurred cyathium correlates with an increased diversification rate, we evaluated the rate of species diversification of the two major subclades of the Pedilanthus clade (PT and core Pedilanthus) relative to the inferred sister to the Pedilanthus

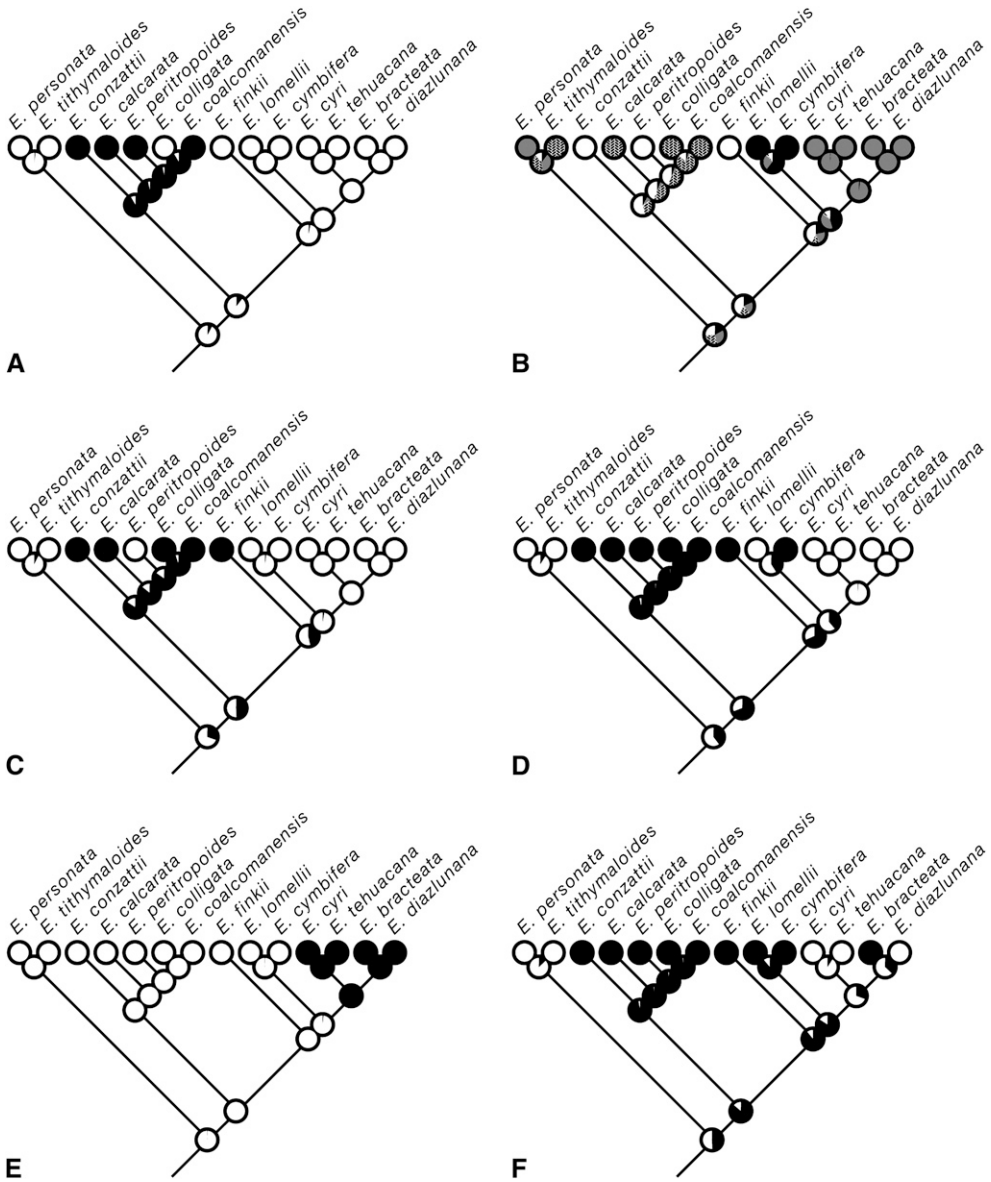

Fig. 8. Maximum likelihood (ML) mapping of six selected morphological traits (only the ingroup is shown). (A) Habit: shrub (white), tree/treelet (black). (B) Habitat: mesophytic (white), TDF (hatched), xeric (gray), desertic (black). (C) Vessel element grouping: solitary (white), grouped (black). (D) Intervessel pitting: scalariform-pseudoscalariform (white); alternate-opposite dominant (black). (E) Latex color: white (white), yellow (black). (F) Spur projection: conspicuous (black), inconspicuous (white).

group. We used the program LRDiverse, which models diversification as a pure birth process and has the advantage over simple comparisons of the numbers of species between sister clades that it takes into account the relative ages of the crown and stem nodes to evaluate alternative models for changes in diversification rate. Relative nodal ages and confidence intervals on those ages were similar for all three alternative dating methods (LF, NPRS, and PL).

As summarized in Table 2, the preferred model depended heavily on whether we count the diversified subspecies of *E. tithymaloides* as being species and on how many species are inferred to be in the sister group to the Pedilanthus clade. The model of diversification that is predicted under the key innovation hypothesis requires that the diversification rate for the two ingroup clades differs (higher) relative to that of the outgroup. The *P*-values corresponding to models of increased complexity successively compared to the "unconstrained" model (each branch its own rate) are shown in Table 2. It can be seen that in all cases that the "outgroup vs. ingroups 1+2" model is no worse than the unconstrained model (and therefore preferred to that more complex model). However, for most clade size assignments, there is at least one other model that is also favored relative to the unconstrained model. In many cases, the one-rate model is also not significantly different than the unconstrained model and, as the simpler model, is preferred. When *E. tithymaloides* is treated as a single species and when the outgroup is assumed to be just a single species, the data are unable to distinguish whether an increase in diversification rate occurred in the branch subtending the Pedilanthus clade (compatible with the key innovation hypothesis), or the branch subtending the core Pedilanthus clade (suggesting an increased diversification rate in the core Pedilanthus subclade relative to both the PT subclade and the outgroup). The only case in which the key innovation hypothesis is uniquely supported (indicated with an asterisk) is when the *E. tithymaloides* subspecies are counted as species and when the sister group comprises just one species.

The Pedilanthus clade is larger in size than its sister group regardless of which of the putative sister groups is considered (one, two, four, or seven species). This fits with the pattern observed by Hodges (1997) that clades bearing spurs contain more species than their spurless sister clades. Adding the Pedilanthus clade to Hodges' (1997) analysis of spurred vs. spurless sister clades, increases the significance of his one-tailed sign test from $P = 0.0352$ to 0.0195. On the other hand, for the Pedilanthus clade, significance under Slowinski and Guyer's (1993) metric for contrasting species numbers is only achieved ($P = 0.0476$) when considering the sister clade as a single species and when the subspecies of *Euphorbia tithymaloides* are treated as individual species.

DISCUSSION

This study contributes to our understanding of traits hypothesized to promote diversification in angiosperms through the analysis of the spurred cyathia, a unique feature of the Pedilanthus clade that is analogous to floral nectar spurs. The evolution

TABLE 2. Shift in diversification rate in the Pedilanthus clade inferred using LRDiverse. We considered four alternative clade sizes for the outgroup: 1, 2, 4, or 7 species. We also considered two ways of counting species within the ingroup: using the traditional species limits (upper part of the table) or treating the recognized subspecies as species (lower part of table). The *P*-values evaluate whether the model explains the data significantly worse than the unconstrained three-rate model. *P*-values less than 0.95 (boldface) suggest that the corresponding simpler model is preferred. The range of *P*-values corresponds to the upper and lower confidence intervals of relative nodal ages for each of three molecular dating methods: Langley-Fitch molecular clock (LF), nonparametric rate smoothing (NPRS), penalized likelihood (PL). A one- or two-rate model is considered uniquely supported (indicated with an asterisk) when it is the only model that is found to not be significantly worse than the unconstrained model.

Out group	Ingroup 1 (core)	Ingroup 2 (PT)	Dating method	One-rate model	Two-rate models (clade subtended by the branch where the shift in rate is inferred)		
					Pedilanthus clade	Core Pedilanthus subclade	PT Pedilanthus subclade
E. tithymaloides treated as one species							
1	12	2	LF	0.992–0.997	**0.779–0.787**	**0.913–0.946**	0.993–1.0
			NPRS	0.988–0.997	**0.79–0.809**	**0.881–0.956**	0.994–1.0
			PL	0.997–0.999	**0.778–0. 8**	**0.941–0.952**	0.999–1.0
2	12	2	LF	**0.925–0.968**	**0.773–0.807**	**0.396–0.402**	0.955–0.982
			NPRS	**0.906–0.959**	**0.775–0.81**	**0.396–0.448**	0.942–0.978
			PL	**0.965–0.996**	**0.781–0.814**	**0.436–0.521**	0.994–1
4	12	2	LF	**0.827–0.899**	**0.797–0.798**	0	0.903–0.973
			NPRS	**0.797–0.882**	**0.767–0.79**	0	0.877–0.954
			PL	**0.883–0.98**	**0.767–0.772**	**0–0.337**	0.955–0.999
7	12	2	LF	**0.739–0.856**	**0.773–0.785**	0	0.826–0.987
			NPRS	**0.713–0.808**	**0–0.735**	0	0.761–0.902
			PL	**0.801–0.96**	**0.764–0.799**	**0**	0.902–0.993
E. tithymaloides subspecies each treated as species							
1	12	9	LF	0.978–0.994	**0 ***	0.989–1.0	0.995–1.0
			NPRS	0.966–0.993	**0 ***	0.983–1.0	0.992–0.995
			PL	0.992–1.0	**0 ***	0.984–1.0	0.999–1.0
2	12	9	LF	**0.903–0.949**	**0**	0.947–0.978	0.958–0.992
			NPRS	**0.849–0.941**	**0**	0.887–0.948	0.934–0.982
			PL	**0.943–0.999**	**0**	0.942–1	0.992–1
4	12	9	LF	**0.768–0.884**	**0**	0.813–0.904	0.88–0.959
			NPRS	**0.709–0.863**	**0**	0.757–0.9	0.857–0.945
			PL	**0.864–0.988**	**0**	0.886–0.995	0.932–0.998
7	12	9	LF	**0.628–0.816**	**0**	0.684–0.843	0.799–0.909
			NPRS	**0.546–0.796**	**0**	0.621–0.812	0.73–0.889
			PL	**0.773–0.964**	**0**	0.784–0.969	0.872–0.988

of other morphological traits in the group was also examined and we used the inferred phylogenetic relationships to shed light on general aspects of the evolution of this small yet diverse clade of *Euphorbia.*

Systematics of the Pedilanthus clade*—Species monophyly and infraspecific discordance* —We recovered a general pattern of species exclusivity in our data. Three instances of discordance were observed and will be discussed in turn.

Euphorbia calcarata—The monophyly of *Euphorbia calcarata* is highly supported in all analyses performed. However, there is incongruence in the relationships of individual alleles, especially when comparing *G3pdhC-A* and ITS (Fig. 6A, 6C). One individual, *E. calcarata*_01, from Chiapas, was found to be a heterozygote at *G3pdhC-A*. One of its alleles (*E. c.*_01a) is closely related to the alleles from accessions of Chiapas and Guatemala (*E. c.*_04, and *E. c.*_06), confirming a southern clade. However, the second allele (*E. c.*_01b) forms a clade with more northern accessions [*E. c.*_02 (Colima), *E. c.*_03 (Michoacán)]. The genetic distinction between northern and southern accessions of *E. calcarata* is also supported by ITS, while *matK* only shows weak support for the northern clade. A second source of conflict between the *G3pdhC-A* and ITS data sets is in the placement of *E. calcarata*_06 (Guatemala). The *G3pdhC-A* gene tree shows *E calcarata*_06 in the southern clade, which is concordant with biogeography, whereas ITS places it in the northern clade. Given the limited seed dispersal ability of these taxa and the great distances involved, it seems most likely that alleles having discordant patterns reflect incomplete lineage sorting.

Euphorbia diazlunana and E. bracteata—G3pdhC-A showed a lack of reciprocal monophyly for *E. diazlunana* (Jalisco) and *E. bracteata* (Sinaloa). Considering the geographical proximity of these species, one might be tempted to invoke recent introgression. However, given that these two taxa appear to have diverged from common ancestry only recently and appear to have premating barriers to gene flow (*E. bracteata* is presumed to be pollinated by hummingbirds, and *E. diazlunana* by hymenopterans; Sahagún-Godínez and Lomelí-Sención, 1997; N. I. Cacho, personal observations), we believe that incomplete lineage sorting is a better explanation for the pattern observed.

Euphorbia tithymaloides and E. personata—The exclusivity of *E. tithymaloides* is supported by ITS (MLBS = 72), and this support increases in the combined-one data set (MLBS = 82). Nonetheless, the *matK* tree suggests some introgression or incomplete lineage sorting between *E. tithymaloides* and *E. personata*. Furthermore, the combined data set with multiple accessions per species fails to resolve *E. personata* as monophyletic.

Despite the lack of consistent reciprocal monophyly, there is morphological (vegetative and reproductive) and ecological evidence of differentiation. *Euphorbia personata* has a discontinuous distribution, with known populations restricted to the northernmost portion of the Yucatan Peninsula, Honduras, and the Santa Rosa National Park in Costa Rica. The populations in Honduras were not studied, but at the other two localities, *E. tithymaloides* also occurs in nearby sites. It is notable that while there are soil differences, in both cases the *E. personata* plants occur in relatively dry deciduous forests, whereas *E. tithymaloides* occurs in more inland forests that have a higher proportion of evergreen species.

Morphologically, *E. personata* is distinguished by having two exposed lateral glands such that the cyathium resembles a face with two eyes (Fig. 3C). These exposed glands make nectar more accessible to insects and might account for floral visits by both hummingbirds and bees (I. N. Cacho, personal observation). This contrasts with *E. tithymaloides* cyathia of nearby populations, which seem to receive almost exclusively hummingbird visits. Also, when entering the staminate phase, the style bends backward to a much greater extent in *E. personata* than in *E. tithymaloides*. The fruit in *E. personata* is densely tomentose (Fig. 3D), whereas in *E. tithymaloides* it is mostly glabrous. Vegetatively, *E. personata* is distinguishable from *E. tithymaloides* by its glaucous, erect, and mostly leafless stems that form more upright shrubs, noticeably taller than plants of *E. tithymaloides* (Fig. 2). The leaves of *E. tithymaloides* are much larger, much glossier, and notably less puberulent than those of *E. personata*. Given the morphological and ecological distinctiveness of *E. personata* and *E. tithymaloides,* we infer that the lack of resolution in the molecular data reflects a lack of variation or possibly incomplete lineage sorting in the markers used rather than nonmonophyly for much of the genome. However, the possibility of recent gene flow between these two entities cannot be ruled out and remains an interesting topic for future research.

Subclade relationships—Our data show a sister relationship between the PT clade, comprising *E. personata* and *E. tithymaloides*, and the rest of the Pedilanthus clade, which we refer to as the core Pedilanthus clade. In the combined data set, this result gains solid clade support (MLBS = 70; MPBS = 95; PP = 0.95; Fig. 7). Several vegetative and reproductive characters are consistent with this result. Members of the core Pedilanthus clade (M, X, and F) tend to share the trait of having larger cyathia, generally with a well-developed and conspicuous spur (Fig. 8F), in contrast with the smaller cyathia and truncate spurs of the PT subclade. Also, the wood anatomy of the core Pedilanthus clade has been suggested to show a number of derived features (Cacho, 2003; Carlquist, 1975, 2001; Dressler, 1957), for example, the presence of wide and clustered vessels (Fig. 8C), alternate or opposite intervessel pits (Fig. 8D), and uniseriate and homogeneous rays. Other traits that appear to be derived within or at the base of the core Pedilanthus clade include yellow latex (Fig. 8E), large cyme bracts, unicolored cyathia, and inaperturate pollen.

Within the core Pedilanthus clade, both loci of *G3pdhC* show a sister relationship between the F and X clades, while ITS and *matK* fail to resolve any relationship with even moderate support. The *G3pdhC* resolution is well supported in the combined analysis (MLBS = 88; MPBS = 91; PP = 0.99). *Euphorbia finkii*, the only member of the F clade, is an evergreen, woody (81% xylem), mesic shrub with large, glabrous, glossy leaves and a spur that is bent forward (Fig. 3L). While represented by a single individual in three of the four markers analyzed, the morphology and ecology of *E. finkii* suggest reproductive and ecological isolation from closely related entities and give no reason to question its monophyly.

The six species that constitute the X clade (MLBS = 100; MPBS = 100; PP = 1.0) are all succulent shrubs that inhabit deserts, thorn scrubs, and tropical deciduous forests. These taxa are either practically leafless (*E. lomelii*, *E. cymbifera*), or markedly deciduous (*E. diazlunana*, *E. bracteata*, *E. cyri*, *E. tehuacana*), and leaves, when present, are mostly densely pubescent. Both of our combined analyses resolve relationships within the xeric clade with reasonably good support, despite a

lack of resolution in the individual markers. The desert-inhabiting species *E. lomelii* and *E. cymbifera* form a moderately supported clade (MLBS = 76; MPBS = 71; PP = 0.94) that is sister to a core xeric clade (MLBS = 100; MPBS = 100; PP = 1.0). Both *E. lomelii* and *E. cymbifera* exhibit some sort of underground dispersion (root adventitious buds in *E. lomelii* and rhizomes in *E. cymbifera*) and have heavily cutinized, glaucous stems.

Two clades of two species comprise the core xeric subclade. *Euphorbia bracteata* is distributed along the interior slopes of the Sierra Madre Oriental, with disjunct populations from Sinaloa to Guerrero. Its sister species (MLBS = 93; MPBS = 95; PP = 1.0), *E. diazlunana*, has a restricted distribution in the Sierra de Manantlán area of Jalisco. In spite of their shared, succulent, shrubby habit, these two taxa are morphologically distinct. *Euphorbia bracteata* is taller, with thicker stems and has brightly colored, persistent bracts that enclose a bright green cyathium with prominent spur and involucral tube. *Euphorbia diazlunana*, in contrast, is a shorter shrub with thinner but more numerous stems (Fig. 2A), has much smaller green bracts, and both the spur and the involucral tube are short and pale (Fig. 3E, 3F). These traits suggest insect pollination in *E. diazlunana* (Sahagún-Godínez and Lomelí-Sención, 1997).

The final pair of species in the X subclade is *E. tehuacana* and *E. cyri*, which form a clade that receives moderate support in the combined analysis (MLBS = 83; MPBS = 76; PP = 0.97). Both species occur in flat scrubland around the city of Oaxaca, an area whose development places both taxa under threat (Olson et al., 2005). These two species are very similar in habit, although *E. cyri* forms much larger clumps than *E. tehuacana* does. Leaf size and indumentum are also very similar, if not indistinguishable under cultivation. However, these two species differ in cyathial morphology. *Euphorbia cyri* has reddish and persistent bracts that enclose its cyathium, whose involucral tube and spur are quite prominent, the spur lobes in this species are fused to a degree that suggests that access to the nectar chamber requires some considerable force. In contrast, *E. tehuacana* has a shortened involucral tube and a spur whose lobes do not enclose the gland chamber as tightly as those of *E. cyri*. Additionally, the style in *E. tehuacana* is shorter and bent back toward the gland chamber (rather than projecting forward), and the staminate flowers are only shortly exserted beyond the involucral tube. Given that a sister relationship between *E. diazlunana* and *E. tehuacana* is convincingly rejected by a Templeton test ($P = 0.0143$) and that such a relationship is not present in any of the trees retained in the Bayesian posterior distributions, the similarity of the cyathia of *E. tehuacana* and *E. diazlunana* could reflect independent transitions from bird to insect pollination.

All five members of the M clade (MLBS = 100; MPBS = 99; PP = 1.0) are woody, with a high percentage of xylem in their stems (average 67% vs. 44% in the X subclade). The species in this clade vary from woody shrubs (*E. colligata*) or treelets (*E. conzattii*, *E. peritropoides*), to true trees (*E. calcarata*, *E. coalcomanensis*). Our data support a sister relationship of *E. conzattii* with the rest of the M clade (MLBS = 90; MPBS = 94; PP = 1.0). This taxon is the most restricted in distribution, with a single population of about 20 individuals at the very top of a single mountain in southwestern Mexico (Olson et al., 2005). Individuals of *E. conzattii* are evergreen treelets about 1 m tall that have caducous cyathial bracts and bright red cyathia (Fig. 3O) that contrast very prominently with the dark green of the surrounding vegetation. The type specimen for this taxon is mixed with material of *E. calcarata* (Dressler, 1957; Olson et al., 2005), but our data show no evidence of introgression or incomplete lineage sorting between these two taxa. On the basis of our field observations and the data here presented, we believe that this taxon is a distinct morphological and genetic entity.

Our data are unable to resolve the relationships among *E. peritropoides*, *E. calcarata*, and the two-species clade formed by *E. colligata* and *E. coalcomanensis* within the core mesic clade. All markers but *G3pdhC-A* resolve *E. peritropoides* as a monophyletic entity with high support, and not surprisingly, the combined-all data do so as well. *Euphorbia peritropoides* is an understory treelet of mesic, seasonal forests with glabrous and glossy leaves and a light pink to bright red spur, with an extremely reduced, green involucral tube and no bracts. Unlike all other taxa in the Pedilanthus clade, cyathia in *E. peritropoides* are borne on pendent inflorescence shoots, each with several cyathia (Fig. 3P).

Euphorbia coalcomanensis, a tree of tropical deciduous forests, is well supported as sister (MLBS = 100; MPBS = 99; PP = 1.0) to *E. colligata*, the only woody shrub of the mesic clade. *Euphorbia coalcomanensis* has densely puberulent leaves that are somewhat succulent (Fig. 2P). In contrast, *E. colligata* leaves are completely glabrous and coriaceous, much like the leaves of the oak forests in which it grows. These two sister species differ in cyathial characteristics as well: cyathia of *E. coalcomanensis* are green with brightly colored and persistent bracts (Fig. 3R), whereas those of *E. colligata* are red with caducous bracts (Fig. 3M).

Relationships of the Pedilanthus clade—Traditionally, sampling issues (both taxonomic and of molecular characters) have played a role in the persistence of unresolved phylogenetic relationships among New World members of the clade C of *Euphorbia*. In addition to the long branch subtending the Pedilanthus clade, sampling issues might contribute to the uncertainty regarding the sister group of the Pedilanthus clade. Our results do not show conclusive support for a Mexican origin for the Pedilanthus clade, as was suggested by Dressler (1957). The PT subclade, which is sister to the rest of the Pedilanthus clade, includes one Mesoamerican species (*E. personata*) and one species that occurs throughout Central America, coastal northern South America and the Caribbean (*E. tithymaloides*). Furthermore, many of the putative closest relatives of the Pedilanthus clade [*E. sinclairiana*, *E. comosa*, *E. pteroneura*, *E. hoffmanniana* (Klotzsch & Garcke) Boiss., *E. weberbaueri* Mansf., *E. cestrifolia* Kunth, *E. calyculata* Kunth, *E. lagunillarum* Croizat, *E. tanquahuete* Sessé & Moc.], occur either in Mexico, Central America, the Caribbean, or northern South America (Steinmann et al., 2007). While a Mexican and even a Caribbean origin of the group remains plausible, it seems likely that the Pedilanthus group had a Central American ancestor that later diversified in central Mexico giving rise to the core Pedilanthus clade. More precise inferences about the phylogenetic relationships of the Pedilanthus clade and its close relatives require a comprehensive study of clade C of *Euphorbia*, with special emphasis on its New World members. Our results suggest that *G3pdhC* might prove to be an excellent marker for such an expanded study. Whether the Pedilanthus clade diversified from a succulent or woody ancestor is not certain at this time because many of the New World clades that are closely related to the Pedilanthus clade present varying degrees of succulence (e.g., *E. pteroneura* and *E. gollmeriana*), while others are rather woody (*E. sinclairiana*).

Character evolution in the Pedilanthus clade—We explored character evolution in the Pedilanthus clade to identify morphological synapomorphies for its different subclades. Our ML

reconstructions suggested that some vegetative characters including habit, vessel grouping, or intervessel pit morphology (Fig. 8A, 8C, 8D) might be quite good predictors of phylogeny at a coarse scale. Other characters might be helpful "locally." For example, yellow latex is a synapomorphy unifying the core xeric clade (Fig. 8E).

There is high homoplasy within reproductive morphological traits and thus a clear absence of simple cyathial synapomorphies for any particular clade. Involucre color, spur size, spur projection (Fig. 8F), spur shape and color, bract morphology (persistence, size, coloration), and gland exposure all contribute to the striking morphological variation of the zygomorphic cyathium in the Pedilanthus clade. Three distinct strategies seem to serve the same ecological function of rendering the cyathium visually conspicuous (equivalent to floral display): (1) numerous small cyathia with brightly colored involucral tubes and truncate spurs; (2) few, large, usually green cyathia with well-developed spurs and involucral tubes and large, persistent, colorful bracts; and (3) few, large cyathia with brightly colored spurs and involucral tubes but inconspicuous, often caducous bracts. Strategy one is found in *E. tithymaloides* and *E. personata* of the PT subclade; in the X and M clades, there is a mixture of strategies two and three. For example, large, persistent, colored bracts are present in *E. coalcomanensis*, *E. bracteata*, *E. cyri*, and *E. tehuacana*, with a maximum of four mature cyathia at a given time (e.g., Fig. 3A), in contrast to 8–12 in the PT clade (Fig. 3C). In cases where there is a single mature cyathium at a given time (e.g., *E. conzattii*, *E. cymbifera*, *E. calcarata*), cyathia have well-developed spurs and involucral tubes, both solidly and brightly colored with caducous bracts (e.g., Figs. 3I, 3K, 3O, 3N). Altogether, character-mapping experiments reveal a lack of defining synapomorphies for the major subclades of the core Pedilanthus clade and suggest that reproductive morphology is more labile than vegetative morphology in the group, as might be expected if a reproductive trait, the cyathial spur, has served as a key innovation.

Is the spurred zygomorphic cyathium a key innovation?—There is now a body of evidence that supports that nectar spurs tend to increase diversification rate. This has been established both with one-tailed sign tests (Hodges, 1997) and with analyses using independent contrasts on a comprehensive supertree of angiosperm families (Kay et al., 2006). This repeated pattern, combined with a plausible causal hypothesis of how nectar spurs might influence diversification, support the claim that floral nectar spurs are key innovations (Hodges, 1997; Kay et al., 2006). Here we explored whether this inference can be extended to the extrafloral nectar spur found in the Pedilanthus clade, which serves an analogous function to that of floral nectar spurs in other angiosperms.

In the Pedilanthus clade, the nectar glands are tightly enclosed within a chamber, the spur, in such a way that full access to the reward (nectar) requires forceful entry by a hummingbird or large insect. Together with the strong zygomorphy of the Pedilanthus cyathium, it has been argued that the presence of nectar glands tightly enclosed in the cyathial spur has opened the adaptive zone of hummingbird pollination in *Euphorbia*, a group that is otherwise mostly insect-pollinated (Dressler, 1957). Observations of plants of the Pedilanthus clade in the wild suggest that hummingbirds play a role in pollen transfer by contacting male and female flowers when probing spurred cyathia for nectar. A high diversity in spur morphology coupled with low sequence variation in the Pedilanthus clade compared to its outgroups, and a pattern of a long branch followed by a sudden diversification in the group, are suggestive of a rapid radiation in the Pedilanthus clade after the evolution of the nectar spur.

In the light of our current knowledge of phylogenetic relationships among the New World members of the Clade C of *Euphorbia*, the most likely sister group to the Pedilanthus clade includes from one to seven taxa (Steinmann and Porter, 2002; Steinmann et al., 2007; V. W. Steinmann, unpublished data). Using the approach of Sanderson and Donoghue (1994), which considers rates of diversification in a relative temporal framework, we found support for a model consistent with the spurred cyathium being a key innovation only when we assumed that the sister group to the Pedilanthus clade is a single species and when the subspecies of *E. tithymaloides* are treated as species (Table 2). Under other scenarios, the data are compatible with a key innovation hypothesis but do not uniquely support it over alternative models. These results are in complete agreement with Slowinski and Guyer's method: significance ($P = 0.0476$) is only achieved when the sister clade to the Pedilanthus clade is a single species and when the subspecies of *E. tithymaloides* are treated as species. On the other hand, the Pedilanthus clade is larger than its sister group regardless of which of the putative sister groups is considered and whether the subspecies of *E. tithymaloides* are granted species status. Thus, adding the Pedilanthus clade to Hodges' (1997) analysis of spurred vs. spurless sister clades, increases the significance of his one-tailed sign test from $P = 0.0352$ to 0.0195.

Our analysis of nectar spurs as key innovations faces two major limitations. One is the relatively small size of the Pedilanthus clade, which is illustrated by a lack of significance when only clade species numbers are taken into account. The minimal species proportion between sister clades to identify a significant directional shift in species diversity based on clade species numbers alone has been shown to be 20 : 1 (Slowinski and Guyer, 1993). The other limitation is an unequal density of sampling of the Pedilanthus clade and its close relatives: while our sampling of members of the Pedilanthus clade is virtually complete, our sampling of outgroups is quite sparse. This unequal sampling density poses serious limitations for methods of analysis that take into account the length of branches in all parts of the tree (e.g., Ree, 2005; Maddison et al., 2007).

We are optimistic that it will be possible to implement approaches with higher statistical power than the one used here in the near future, as new information on the phylogenetic relationships within *Euphorbia* comes to light, especially with respect to the New World members of the clade C of *Euphorbia*. Also, improved knowledge of *Euphorbia* phylogenetics will make it possible to statistically test the role of other traits that have been proposed to increase the rate of diversification in this huge clade of flowering plants. For example, the cyathium itself has been proposed as a key innovation explaining the tremendous diversification of *Euphorbia* (Steinmann and Porter, 2002; Prenner and Rudall, 2007; Prenner et al., 2008). This hypothesis seems plausible considering that *Euphorbia* is the second largest plant genus (ca. 2100 species) and is sister to a relatively species-poor group, Neoguillauminiinae (six species), which lacks cyathia.

This study was not intended as a test of a causal hypothesis but rather as an evaluation of whether a general correlation found for floral nectar spurs and increased diversification rates might hold for an analogous structure, the cyathial spur of Pedilanthus. In *Aquilegia*, pollinator shifts drive diversification by imposing directional selection on nectar spurs (Whittall and Hodges, 2007). In the case of the Pedilanthus clade, our analy-

sis neither supports nor rejects the hypothesis that pollinator shifts have played a role in promoting diversification in this group. Slight changes in the morphology of the spur of Pedilanthus could either cause shifts in pollinator identity, whether to different hummingbird species or, in two cases, to insect pollinators, or it could alter the location of pollen deposition on a specific pollinating species and thus increase the rate at which premating reproductive isolation can evolve. Future pollination studies of the Pedilanthus clade will allow these causal hypotheses to be tested. Also, future systematic research that enables the use of more powerful methods, clarifies the composition of the sister group to the Pedilanthus clade, and reevaluates the status of infraspecific taxa within *E. tithymaloides* could lead to a more definitive statement as to whether the evolution of the spurred cyathium correlates with increased species diversification. However, by analogy to floral spurs, and allowing for the fact that the Pedilanthus clade could be too young a group to show statistically significant evidence of increased diversification under the methods here applied, it remains plausible that the cyathial nectar spur of the Pedilanthus clade of *Euphorbia* has spurred the diversification of this distinctive group of spurges.

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APPENDIX 1. Voucher and GenBank accession information for specimens included in this study. Identification number, locality information, and herbaria are provided for specimens collected for this study; sequences downloaded from GenBank are identified as "downloaded"). Abbreviations: DAV= Herbarium at University of California-Davis; HAJB = Jardín Botánico de la Habana, Cuba; MEO = Mark E. Olson; MEXU = Herbario Nacional de México; NIC = N. Ivalú Cacho; UCB = University of California-Berkley Botanical Garden; UCD = University of Californinia-Davis Botanical Garden; UC/JEPS: The University and Jepson Herbaria at University of California-Berkeley; UWBot = University of Wisconsin-Madison Botany Greenhouses; UWDCS = University of Wisconsin-D. C. Smith Greenhouse; WIS = Wisconsin State Herbarium.

Taxon, *Voucher* (or other identification number), Locality, Herbaria, GenBank accessions *G3pdhC-A*, *G3pdhC-B*, ITS, *matK*

Ingroup—***E. bracteata_1***, *MEO & NIC 845*, México: Sinaloa, WIS, MEXU, [GU214886 (direct sequencing), GU214892 (clone 3)], GU214954, GU214909, n/a; ***E. bracteata_2***, *MEO & NIC 1011*, México: Guerrero, WIS, MEXU, GU214887, GU214967, GU214910, GU214846; ***E. bracteata_3***, *MEO & NIC 1010*, México: Guerrero, WIS, MEXU, GU214888, GU214976, GU214911, GU214854; ***E. bracteata_4***, downloaded, n/a, n/a, n/a, n/a, AF537489.1, n/a; ***E. calcarata_1***, *MEO 806*, México: Chiapas, WIS, MEXU, [GU214895 (clone 4), GU214896 (clone 9)], GU214957, GU214912, GU214835; ***E. calcarata_2***, *MEO & NIC 896*, México: Colima, WIS, MEXU, GU214874, GU214962, GU214913, GU214840; ***E. calcarata_3***, *MEO & NIC 900*, México: Michoacán, WIS, MEXU, GU214875, GU214963, GU214914, GU214841; ***E. calcarata_4***, *MEO & NIC 939A*, México: Chiapas, WIS, MEXU, GU214897, GU214964, GU214915, GU214843; ***E. calcarata_5***, *MEO & NIC 939*, México: Chiapas, WIS, MEXU, n/a, GU214969, GU214916, GU214848; ***E. calcarata_6***, *NIC 407*, Guatemala: Nentón, WIS, GU214883, GU214980, GU214917, GU214857; ***E. calcarata_7***, downloaded, n/a, n/a, AF537492.1, n/a; ***E. coalcomanensis***, *MEO & NIC 886*, México: Michoacán, WIS, MEXU, GU214873, GU214961, GU214918, GU214839; ***E. colligata_1***, *MEO & NIC 866*, México: Jalisco, WIS, MEXU, [GU214870 (direct sequencing), GU214889 (clone 9)], GU214958, GU214919, GU214836; ***E. colligata_2***, *MEO & NIC 867*, México: Jalisco, WIS, MEXU, [GU214871 (direct sequencing), GU214890 (clone 2), GU214891 (clone 3)], GU214959, GU214920, GU214837; ***E. colligata_3***, *MEO & NIC 867A*, México: Jalisco, WIS, MEXU, GU214872, GU214960, GU214921, GU214838; ***E. colligata_4***, downloaded, n/a, n/a, AF537493.1, n/a; ***E. conzattii***, *MEO & NIC 971*, México: Oaxaca, WIS, MEXU, GU214880, GU214972, GU214922, GU214851; ***E. cymbifera***, *MEO & NIC 979*, México: Puebla, WIS, MEXU, GU214869, GU214956, GU214923, GU214834; ***E. cymbifera_1***, downloaded, n/a, n/a, AF537491.1, n/a; ***E. cyri***, *MEO & NIC 973*, México: Oaxaca, WIS, MEXU, GU214894, n/a, GU214926, GU214833; ***E. diazlunana***, *MEO & NIC 888*, México: Jalisco, WIS, MEXU, [GU214893 (clone 4), GU214901 (clone 1)], GU214968, GU214927, GU214847; ***E. finkii***, *MEO & NIC 917*, México: Oaxaca, WIS, MEXU, GU214898, GU214973, GU214929, GU214852; ***E. finkii_1***, downloaded, n/a, n/a, AF537520.1, n/a; ***E. lomelii_4***, downloaded, n/a, n/a, AF537490.1, n/a; ***E. lomelii_1***, *MEO & NIC 852*, México: BCS, WIS, MEXU, GU214868, GU214955, GU214933, GU214831; ***E. lomelii_2***, *UCD 99263*, México: BCS, DAV, GU214885, GU214981, GU214934, GU214860; ***E. lomelii_3***, *UCB 62.0776*, México: BCS, UC/JEPS, n/a, n/a, GU214935, n/a; ***E. peritropoides_1***, *MEO & NIC 974*, México: Oaxaca, WIS, MEXU, GU214877, GU214966, GU214937, GU214845; ***E. peritropoides_2***, *MEO & NIC 996*, México: Guerrero, WIS, MEXU, GU214878, GU214970, GU214938, GU214849; ***E. personata_1***, *MEO & NIC 955*, México: Yucatán, WIS, MEXU, GU214899, GU214974, GU214939, GU214832; ***E. personata_2***, *NIC 343*, Guatemala, WIS, n/a, GU214979, GU214940, GU214856; ***E. t. subsp. angustifolia_1***, *NIC 059.2*, USA: USVI: St. John, WIS, GU214881, GU214977, GU214945, GU214855; ***E. t. subsp. angustifolia_2***, *NIC 073.2*, Dominican Republic, WIS, GU214882, GU214978, GU214946, n/a; ***E. t. subsp. padifolia***, *Iltis 30229*, Statia, WIS, GU214879, GU214971, GU214947, GU214850; ***E. t. subsp. tithymaloides_1***, *MEO & NIC 926*, México: Oaxaca, WIS, MEXU, n/a, n/a, GU214948, GU214842; ***E. t. subsp. tithymaloides_2***, *MEO & NIC 947*, México: Oaxaca, WIS, MEXU, GU214876, GU214965, GU214949, GU214844; ***E. t. subsp. tithymaloides_3***, *NIC 139*, Guatemala: Cuija, WIS, GU214884, n/a, GU214950, GU214858; ***E. t. subsp. tithymaloides_4***, *NIC 140*, Guatemala: Cahuí, WIS, n/a, n/a, GU214951, GU214859; ***E. t. subsp. tithymaloides_5***, downloaded, n/a, n/a, AF537494.1, n/a; ***E. tehuacana***, *MEO & NIC 981*, México: Puebla, WIS, MEXU, GU214900, GU214975, GU214944, GU214853; ***E. tehuacana_1***, downloaded, n/a, n/a, AF537488.1, n/a

Outgroups—***E. cestrifolia*** Kunth, downloaded, n/a, n/a, AF537521.1, n/a; ***E. comosa*** Vell., downloaded, n/a, n/a, AF537503.1, n/a; ***E. cymosa*** Poir., *NIC 083*, Jamaica, WIS, GU214906, GU214988, GU214924, GU214865; ***E. cyparissias*** L., *NIC 429*, USA: Wisconsin, WIS, GU214908, GU214984, GU214925, GU214866; ***E. esula*** L., *NIC 428*, USA: Wisconsin, WIS, GU214907, GU214985, GU214928, n/a; ***E. gollmeriana*** Klotzsch ex Boiss., *NIC 126*, Venezuela: Falcón, WIS, GU214904, GU214982, GU214930, n/a; ***E. heterophylla*** L., *NIC 044*, Puerto Rico: Manatí, WIS, n/a, n/a, GU214931, GU214861; ***E. hoffmanniana*** (Klotzsch & Garcke) Boiss., downloaded, n/a, n/a, AF537508.1, n/a; ***E. humifusa*** Willd., downloaded, n/a, n/a, n/a, AB233780.1; ***E. leucocephala*** Lotsy, *NIC 414*, Guatemala: Nentón, WIS, GU214902 (clone 4), GU214986, GU214932, GU214862; ***E. lindenii*** (S.Carter) Bruyns, downloaded, n/a, n/a, AF537473.1, n/a; ***E. milii*** Des Moul., *NIC 626*, UWBot, WIS, GU214903 (clone 2), GU214987, GU214936, GU214864; ***E. obesa*** Hook.f., downloaded, n/a, n/a, AF537566.1, n/a; ***E. petiolaris*** Sims, *NIC 054*, USA: USVI: St. John, WIS, n/a, n/a, GU214941, n/a; ***E. polyacantha*** Boiss., downloaded, n/a, n/a, n/a, AY491656.1; ***E. pteroneura*** A.Berger***_1***, *NIC 411*, Guatemala: Nentón, WIS, n/a, n/a, GU214942, GU214867; ***E. pteroneura _2***, downloaded, n/a, n/a, AF537506.1, n/a; ***E. pulcherrima*** Willd. ex Klotzsch***_1***, *NIC 406*, Guatemala:Petén, WIS, n/a, n/a, GU214943, n/a; ***E. pulcherrima _2***, downloaded, n/a, n/a, AF537432.1, n/a; ***E. sinclairiana*** Benth., downloaded, n/a, n/a, AF537495.1, n/a; ***E. umbellata*** (Pax) Bruyns, downloaded, n/a, n/a, AF537469.1, AB233784.1; ***E. umbelliformis*** (Urb. & Ekman) V.W.Steinm. & P.E.Berry, *HAJB 81901*, Cuba, WIS, GU214905, GU214983, GU214952, n/a; ***E. weberbaueri*** Mansf., downloaded, n/a, n/a, AF537519.1, n/a; ***Manihot esculenta***, *NIC 625*, UWDCS, WIS, n/a, n/a, GU214953, GU214863; ***Neoguillauminia cleopatra*** (Baill.) Croizat, downloaded, n/a, n/a, AF537581.1, n/a.