

A Rich Fossil Record Yields Calibrated Phylogeny for Acanthaceae (Lamiales) and Evidence for Marked Biases in Timing and Directionality of Intercontinental Disjunctions

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Abstract.—More than a decade of phylogenetic research has yielded a well-sampled, strongly supported hypothesis of relationships within the large (>4000 species) plant family Acanthaceae. This hypothesis points to intriguing biogeographic patterns and asymmetries in sister clade diversity but, absent a time-calibrated estimate for this evolutionary history, these patterns have remained unexplored. Here, we reconstruct divergence times within Acanthaceae using fossils as calibration points and experimenting with both fossil selection and effects of invoking a maximum age prior related to the origin of Eudicots. Contrary to earlier reports of a paucity of fossils of Lamiales (an order of ~23,000 species that includes Acanthaceae) and to the expectation that a largely herbaceous to soft-wooded and tropical lineage would have few fossils, we recovered 51 reports of fossil Acanthaceae. Rigorous evaluation of these for accurate identification, quality of age assessment and utility in dating yielded eight fossils judged to merit inclusion in analyses. With nearly 10 kb of DNA sequence data, we used two sets of fossils as constraints to reconstruct divergence times. We demonstrate differences in age estimates depending on fossil selection and that enforcement of maximum age priors substantially alters estimated clade ages, especially in analyses that utilize a smaller rather than larger set of fossils. Our results suggest that long-distance dispersal events explain present-day distributions better than do Gondwanan or northern land bridge hypotheses. This biogeographical conclusion is for the most part robust to alternative calibration schemes. Our data support a minimum of 13 Old World (OW) to New World (NW) dispersal events but, intriguingly, only one in the reverse direction. Eleven of these 13 were among Acanthaceae s.s., which comprises >90% of species diversity in the family. Remarkably, if minimum age estimates approximate true history, these 11 events occurred within the last ~20 myr even though Acanthaceae s.s. is over 3 times as old. A simulation study confirmed that these dispersal events were significantly skewed toward the present and not simply a chance occurrence. Finally, we review reports of fossils that have been assigned to Acanthaceae that are substantially older than the lower Cretaceous estimate for Angiosperms as a whole (i.e., the general consensus that has resulted from several recent dating and fossil-based studies in plants). This is the first study to reconstruct divergence times among clades of Acanthaceae and sets the stage for comparative evolutionary research in this and related families that have until now been thought to have extremely poor fossil resources. [Acanthaceae; BEAST; biogeography; calibration; clade age; comparative; Cretaceous; divergence time estimation; diversification; evolution; fossil; Jurassic; Lamiales; palynology; pollen; simulation; Triassic.]

Across the tree of life, numerous studies from a diversity of lineages have demonstrated the significance of the paleontological record to inference of evolutionary history (Eldridge and Gould 1972; Friis and Endress 1990; Jablonski 1993; Redecker et al. 2000; Crane et al. 2004). Although this record is indisputably incomplete and is plagued by several potential sources of error (Gandolfo et al. 2008), few other satisfactory methods for calibrating phylogenies and reconstructing the timing of major evolutionary events exist. The molecular clock has been refuted for many lineages representing diverse life forms (e.g., Moran 1996; Pereira and Baker 2006; also see Martin and Palumbi 1993) and geological events of sufficient magnitude and scale to provide age calibrations are limited (e.g., island archipelagos; Hormiga et al. 2003; Emerson and Oromí 2005; but see Heads 2011). Thus, although recent authors have rightfully expressed concern over issues with divergence time estimation (e.g., fossil record incompleteness or quality, Marshall 1990; methodological approaches and model specification, Buckley 2002; Lemmon and Moriarty 2004; Sibon and Drummond 2012), the alternative—ignoring the fossil record altogether—seems unacceptable. Similarly, absent consideration

of fossils and their geographic locations, biologists have only phylogenetic hypotheses and modern-day taxon distributions for inferring biogeographic history (Manchester 1999). The abundant fossil evidence of organisms recovered in places distant from their current ranges (Manchester 1999; Mayr 2004) highlights the pitfalls of disregarding fossil evidence, even if small in quantity.

The incompleteness of the fossil record has been particularly problematic for research on lineages distributed primarily in low latitudes. It is widely recognized that tropical biomes contribute relatively few fossil specimens to the paleontological record compared to others (Stern and Eyde 1963; Graham 2010). Low fossilization rates in the tropics have been attributed to a paucity of sedimentary rocks and lakes, high rates of erosion (especially for mountainous areas) and rapid decay of organic matter. Fossil record incompleteness is particularly pronounced in flowering plants of tropical latitudes, which are the regions of highest extant species diversity (e.g., approximately one-third of all plant species reside in the Neotropics alone; Gentry 1982; Graham 2010). Clearly, evolutionary research on a sizeable fraction of Earth's total biological

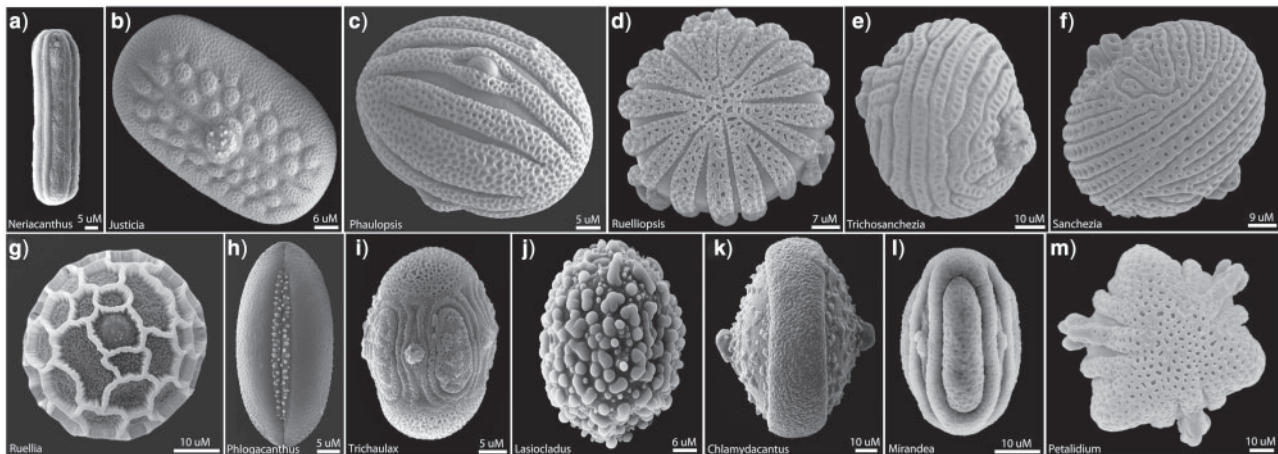


FIGURE 1. Pollen diversity in Acanthaceae, illustrating small fraction of variation found among lineages (see Appendix 1 for further details). *Top row* depicts pollen types relevant to fossils utilized in this study: a) Acanthaceae: *Neriaceanthus grandiflorus* (Daniel et al. 8152), with colpate (simple) apertures. b) Justicieae: *Justicia tenuistachys* (Colque and Tapia 276), showing tricolporate (compound) apertures, with characteristic “insulae” on apertural face. c) Ruellieae: *Phaulopsis betonica* (Love and Congdon 3157), showing tricolporate (compound) apertures surrounded by characteristic “sexine lips” and bands of pseudocolpi between apertures. d) Ruellieae: *Ruelliopsis setosa* (Smith 3107), showing same features as (c). e) Ruellieae: *Trichosanchezia chrysothrix* (Diaz et al. 6954), showing bicolorporate (compound) apertures surrounded by “sexine lips” and bands of pseudocolpi that are arranged in opposing 90° orientations. f) Ruellieae: *Sanchezia decora* (Foster 8790), showing same features as (e). *Bottom row* illustrates additional variation found in the family. g) Ruellieae: *Ruellia geayi* (Daniel 11048). h) Andrographideae: *Phlogacanthus thyrsoiflorus* (Lindberg 200). i) Justicieae: *Trichaulax mwasumbii* (Mwasumbi 14238). j) Barlerieae: *Lasiocladus* sp. (Daniel et al. 11058). k) Whitfieldieae: *Chlamydacanthus euphorbioides* (Capuron 24734P). l) Justicieae: *Mirandea sylvatica* (Wendt et al. 4104). m) Ruellieae: *Petalidium ramulosum* (Volk 57). Images reproduced from SEM micrographs from earlier studies (a: McDade et al. 2005; c–g: Tripp et al. 2013; h, j, k: McDade et al. 2008; i, l: Daniel et al. 2008) except (b) (courtesy of C. Kiel) and (m) (Tripp, unpublished).

diversity has been stymied by a paucity of suitable fossil material.

For low-latitude lineages of flowering plants that *do* have a fossil record, pollen has been a principal information source for testing competing hypotheses of divergence times and evolutionary history (Sauquet et al. 2009; Smedmark et al. 2010; Thornhill et al. 2012) and for reconstructing large-scale patterns of tropical vegetation change (e.g., Burnham and Graham 1999). However, use of this rich information source is limited by taxonomic utility of pollen form: in many plant families, pollen is notoriously invariable (e.g., Poaceae: the grass family). Knowledge of the history and evolution of tropical environments may therefore be enriched by attention to families with taxonomically useful pollen types.

Among the 413 presently recognized flowering plant families (APG III 2009), Acanthaceae have long been appreciated for extreme diversity of pollen forms (Raj 1961; Mabberley 1989; Daniel 1998; Pereira Baker 2000; Scotland and Vollesen 2000; Fig. 1). For example, Raj (1961, p. 69) wrote “Probably no other family has such a wide range of pollen morphological features as the Acanthaceae.” With >4000 species, Acanthaceae are among the 10 or so most diverse families of flowering plants (commonly cultivated species include: bear’s breeches: *Acanthus mollis*; shrimp plant: *Justicia brandegeana*; zebra plant: *Aphelandra squarrosa*; nerve plant: *Fittonia albivensis*; wild petunias: *Ruellia* spp.). Species of Acanthaceae are common constituents of most tropical and subtropical ecosystems, with centers of species-richness in the lower latitudes of the Americas, Africa (including Madagascar) and portions

of Asia. Empirical studies seeking to understand the temporal context for diversification of this and other large, low-latitude plant families will broadly inform investigation of tropical ecosystem histories (Pennington et al. 2000; Pennington et al. 2004).

In addition to contributing more broadly to research in tropical biomes, a major motivation for this study is our documentation of complex geographical distributions among Acanthaceae (e.g., McDade et al. 2005; Daniel et al. 2008; Tripp and McDade 2012; see especially Tripp et al. 2013). Dramatic tectonic events (e.g., continental drift, episodes of mountain building), global climate changes and sea level fluctuations have without doubt contributed to the diversification of Earth’s biota as well as to past and present biogeographical distributions. Acanthaceae, with numerous examples of pantropical distributions, long-distance disjunctions and other intriguing geographical patterns such as sympatry of sister taxa, have clearly written a complex history of diversification on this geological stage. Building a time-calibrated hypothesis of phylogenetic relationships for the group will shed light on the temporal context of this complex history and facilitate finer scale studies of individual clade histories within Acanthaceae.

In the present study, we use nearly 10 kb of DNA sequence data (the largest matrix yet assembled for Acanthaceae) and an extensive database of 51 putative fossil Acanthaceae to (1) investigate the impact on clade ages of inclusion of different sets of fossils that vary in utility (see Methods section); (2) investigate the impact of invoking a maximum age prior on resultant clade ages; (3) infer the temporal origin of Acanthaceae and major

clades within it, with particular attention to lineages that contain both OW and NW constituents; and (4) compare results from our study to previous age estimates for the family. We then use these time-calibrated phylogenies to assess competing biogeographical hypotheses (e.g., Gondwanan vicariance [Raven and Axelrod 1974; Gentry 1982]; land bridge migration [e.g., Tiffney 1985]; long-distance dispersal [Thorne 1973]) to explain OW/NW disjunctions in this species-rich tropical family. The first two scenarios are separated by major temporal gaps (e.g., ≥ 96 Ma [Gondwana] vs. ≥ 65 Ma [numerous land bridges, discussed below]) and therefore may be treated as mutually exclusive; rejection of these two modes generally favors long-distance dispersal scenarios (see, e.g., Dick et al. 2007; Särkinen et al. 2007). The end result is a robust reconstruction of lineage divergence times that underlie the evolution of one of the most diverse families of angiosperms, and one with a surprisingly rich fossil record that has not been exploited to estimate the temporal context of diversity until the present study.

METHODS

Fossils

We conducted an extensive survey of published reports of fossils that have been attributed to Acanthaceae (Table 1). A small number of these original reports have been referenced in prior studies, but there has been no concerted effort to locate and compile all original reports nor have putative fossils been assessed for taxonomic validity in a comprehensive manner. We included unpublished reports and gray literature whenever citations elsewhere led us to them and they were available to us, but did not exhaustively mine unpublished or gray literature. Most reports did not include information as to present location of fossil specimens, thus precluding direct study of them. In most cases, however, images of the fossils were provided and we scrutinized these to assess the accuracy of taxonomic identification. The ages of most fossils were presented as geological time periods that were based on biostratigraphy using characteristic assemblages of index fossils that are understood to have narrow time windows. We used the Geological Time Scale provided by the International Commission on Stratigraphy (2009) to assign age spans in years.

To assess fossil utility for divergence time estimations, we developed an informal ranking system for fossils (guided where possible by recommendations in Parham et al. 2012) ranging from 1 to 5, with 5 indicating highest utility. This ranking reflected (1) our confidence in taxon identification; and (2) our confidence in accuracy of determined age, which was derived from information provided in each publication. We considered 1s and 2s to be low utility reports ($n = 34$), 3s to be medium utility reports ($n = 2$) and 4s and 5s ($n = 8$ and $n = 7$, respectively) to be high utility reports. Low utility fossil reports (1s and 2s) were excluded from further consideration; only 3s, 4s and 5s were employed in this study. These last

were further examined to evaluate the likely usefulness of each fossil in analyses (e.g., extremely young fossils such as those from the Holocene [e.g., fossil #39], or extremely old fossils such as those from the Triassic and Jurassic [i.e., fossils #49, #50] [Table 1] were not used in our analyses, but see Discussion section below). Appendix 1 provides information on these 51 fossils including synapomorphies that link them to modern lineages and justification for assigned utility scores.

Molecule and Taxon Sampling

To estimate phylogenetic relationships and divergence times, we used DNA sequence data from seven markers: one nuclear (ribosomal ITS+5.8S) plus six chloroplast (intron: *rps16*; spacers: *psbA-trnH*, *trnG-trnR*, *trnS-trnG*, *trnL-trnF*, *trnT-trnL*). Most molecular data used in the present investigation were generated previously for earlier studies except for several *trnG-trnR* and *psbA-trnH* sequences and a few *rps16*, *trnT-trnL* and *trnL-trnF* sequences (for laboratory protocols for these loci, see McDade et al. 2005; Tripp 2007; McDade et al. 2008; Tripp 2010). To ensure that taxa from all major lineages of Acanthaceae s.l. were sufficiently sampled, we used McDade et al. (2008) to guide sampling. The final data set consisted of 72 taxa: 3 Nelsonioideae, 3 *Avicennia*, 5 Thunbergioideae, 11 Acantheae, 3 *Neuracanthus*, 9 Whitfieldieae, 12 Barlerieae, 5 Andrographideae, 9 Ruellieae, 10 Justiceae and 2 outgroups (*Sesamum*, *Martynia*). This taxon sample was selective rather than exhaustive; our intention was to represent all major lineages and span the phylogenetic diversity within them, and to compile a data set that would not be prohibitively large for the purposes of reconstructing divergence times. Sampling within each clade was guided by knowledge of phylogenetic relationships based on extensive prior study (McDade et al. 2000a, 2000b; McDade et al. 2005; Kiel et al. 2006; Borg et al. 2008; Daniel et al. 2008; McDade et al. 2008; McDade et al. 2012; Tripp et al. 2013). Within each clade, we ensured that sampled members spanned the basalmost node in order to best approximate true crown clade ages rather than underestimate them (except for the *Neuracanthus* lineage, for which our knowledge of phylogenetic relationships is still limited and we cannot be confident that our taxon sampling captured the basalmost node). For example, within Acantheae, the “one-lipped” and “two-lipped” clades are basally branching sister lineages and members of both lineages were sampled. See Appendix 2 for accession information.

Phylogenetic Analysis

We excluded characters in regions where homology assessment was problematic (e.g., hypervariable regions, apparent SSRs; in total <500 bp) as well as all indels because of model specification limitations in BEAUTi (at present, only nucleotide data, but not binary data, can be analysed). Nearly all nodes were strongly supported regardless of character-exclusion regime (results not

TABLE 1. Reports of fossil Acanthaceae investigated for present study arranged phylogenetically (see Fig. 3)

Taxon	#	Provenance	Geologic period	Utility	Reference
<i>Avicennia</i> (Avicennia lineage)	1	Spain	Middle Bartonian	4	Cavagnetto and Anadón (1996)
<i>Avicennia</i> (<i>Avicennia</i> lineage)	2	Central America	Quaternary	1	Burnham and Graham (1999)
<i>Avicennia</i> (<i>Avicennia</i> lineage)	3	France	Ca. 55 Ma	2	Gruas-Cavagnetto et al. (1987)
<i>Avicennia</i> (<i>Avicennia</i> lineage)	4	Australia	Lower Tertiary	2	MacPhail et al. (1994)
<i>Mendoncia</i> (Thunbergioideae)	5	Central America	Quaternary	1	Burnham and Graham (1999)
Acanthaceae	6	Colombia	Mid to upper Miocene	2	Hooghiemstra et al. (2006)
Acanthaceae	7	Colombia	Mid Pliocene	2	Hooghiemstra et al. (2006)
Acanthaceae	8	India	Tertiary	2	Nair (1968)
Acantheae	9	Senegal	Upper Miocene	2	Medus (1975)
<i>Acanthus</i> (Acantheae)	10	Southeast Asia	Upper Miocene	4	Medley's (1977)
<i>Acanthus</i> (Acantheae)	11	England	Lower to middle Oligocene	4	Reid and Chandler (1926)
<i>Acanthus</i> (Acantheae)	12	China	Pliocene	2	Song et al. (2004)
<i>Blepharis</i> (Acantheae)	13	Namibia	Holocene	1	Gil-Romera et al. (2006)
<i>Blepharis</i> (Acantheae)	14	South Africa	Late Quaternary	2	Scott (1982)
<i>Hulemacanthus</i> (Barlerieae)	15	Nigeria	Miocene	5	Kuyl et al. (1955)
<i>Lepidagathis</i> (Barlerieae)	16	Ethiopia	Upper Miocene	2	Yemane et al. (1987)
Whitfieldieae or Isoglossinae (Justicieae)	17	Southern Africa	Mid Cretaceous	2	Zavada (1992)
Justicieae	18	Senegal	Upper Miocene	4	Medus (1975)
Justicieae	19	Senegal	Upper Miocene	4	Medus (1975)
OW Justicieae	20	Egypt	Lower Miocene	4	Beialy et al. (2005)
<i>Dicliptera</i> (Justicieae)	21	South Africa	Late Quaternary	2	Scott (1982)
<i>Glossochilus</i> (Justicieae)	22	South Africa	Late Quaternary	2	Scott (1982)
<i>Hypoestes</i> (Acantheae)	23	Taiwan	Lower Pliocene	1	Song et al. (2004)
OW <i>Justicia</i> (Justicieae)	24	China	Pliocene	2	Song et al. (2004)
<i>Metarungia</i> (Justicieae)	25	South Africa	Late Quaternary	2	Scott (1982)
<i>Monechma</i> (Justicieae)	26	South Africa	Late Quaternary	2	Scott (1982)
NW <i>Justicia</i> (Justicieae)	27	Central America	Quaternary	1	Burnham and Graham (1999)
NW <i>Justicia</i> (Justicieae)	28	Mexico	Ca. 10–12 Ma	4	Graham (1976)
NW <i>Justicia</i> (Justicieae)	29	Venezuela	Neogene	3	Germeraad et al. (1968)
NW <i>Justicia</i> (Justicieae)	30	Brazil	Lower Miocene	2	Regali et al. (1974)
NW <i>Justicia</i> (Justicieae)	31	Colombia	?	1	van der Hammen (1956)
NW Justicieae	32	Argentina	Mid Miocene	5	Mautino (2011)
OW <i>Justicia</i> (Justicieae)	33	South Africa	Late Quaternary	2	Scott (1982)
OW <i>Justicia</i> / <i>Monechma</i> (Justicieae)	34	Namibia	Holocene	1	Gil-Romera et al. (2006)
pseudocolpate Ruellieae	35	Senegal	Upper Miocene	3	Medus (1975)
pseudocolpate Ruellieae	36	Senegal	Upper Miocene	4	Medus (1975)
<i>Petalidium</i> (Ruellieae)	37	Namibia	Holocene	1	Gil-Romera et al. (2006)
<i>Ruellia</i> (Ruellieae)	38	Namibia	Holocene	1	Gil-Romera et al. (2006)
<i>Ruellia</i> (Ruellieae)	39	Nigeria	Miocene	2	Kuyl et al. (1955)
<i>Ruellia</i> (Ruellieae)	40	Spain	Upper Eocene	2	Cavagnetto and Anadón (1996)
<i>Ruellia</i> (Ruellieae)	41	California, USA	Mid Eocene to lower Oligocene	2	Fredriksen et al. (1983)
Trichantherinae (Ruellieae)	42	Central America	Quaternary	1	Burnham and Graham (1999)
Trichantherinae (Ruellieae)	43	Mexico	Miocene	5	Graham (1976)
Trichantherinae (Ruellieae)	44	Caribbean	Neogene	5	Germeraad et al. (1968)
Trichantherinae (Ruellieae)	45	Trinidad	Tertiary	5	Kuyl et al. (1955)
Trichantherinae (Ruellieae)	46	Brazil	Lower Miocene	2	Regali et al. (1974)
Trichantherinae (Ruellieae)	47	Central America	Quaternary	1	Burnham and Graham (1999)
Trichantherinae (Ruellieae)	48	Arizona, USA	Upper Triassic	2	Pocock and Vasanthy (1988)
Trichantherinae (Ruellieae)	49	France	Upper Jurassic	2	Cornet and Habib (1992)
NW <i>Justicia</i> (Justicieae)	50	Argentina	Mid Miocene	5	Mautino (2011)
NW pseudocolpate Ruellieae	51	Haiti	Miocene–Pliocene	5	Graham (1990)

Notes: Fossils ranked 3, 4 and 5 that were utilized in divergence time estimations are shown in **bold** (see text for explanation of ranking scale for fossil utility; note that not all 3s, 4s and 5s were used because in several cases, more than one fossil was available to constrain a given node). Taxon name refers to crown groups. Fossil ages and calibration priors (lognormal mean, SD and zero offsets) provided in Tables 2 and 3. Appendix 1 provides explanation for ranking assigned to all fossils and synapomorphy-based justification for identification and phylogenetic placement.

shown). Because there were no supported topological differences among individual data partitions (cf. Mason-Gamer and Kellogg 1996; not shown), we concatenated data from the seven markers into a single matrix. This matrix consisted of 9071 nt characters and is available in Dryad (doi: <http://dx.doi.org/10.5061/dryad.jm4d5>).

Testing for Validity of a Molecular Clock

We assessed rate homogeneity among the 72 taxa in our nucleotide data partition by comparing log-likelihoods of trees in which a molecular clock was enforced (null hypothesis) versus not enforced (alternative hypothesis). We used PAUP* (Swofford 2002)

to estimate log-likelihoods, then calculated a likelihood ratio test statistic as twice the difference between the likelihood scores of the two models ($2[\log H_o - \log H_a]$). The result was compared to a chi-squared distribution with $n-2$ degrees of freedom, where n = the number of taxa (Huelsenbeck and Crandall 1997).

Fossil Constraints

To determine the most appropriate placement for fossil constraints in divergence time analyses, we first assessed each fossil for its phylogenetic affinity based on knowledge derived from extensive study of the family. Appendix 1 presents discussion and diagnosis of synapomorphies upon which our assessments are based with references to relevant phylogenetic studies. Most lineages of Acanthaceae have been subject to phylogenetic analysis published in papers that also include identification of morphological synapomorphies for many clades: McDade et al. (2000a), McDade et al. (2000b), Scotland and Vollesen (2000), Schwarzbach and McDade (2002), McDade et al. (2005), Kiel et al. (2006), Tripp (2007), Borg et al. (2008), Daniel et al. (2008), McDade et al. (2008), Tripp et al. (2009), McDade et al. (2012), Tripp et al. (2013). We then used this information to guide the placement of fossil constraints and to create "taxon sets": clades to be constrained by each fossil calibration. Notably, although homoplasious pollen types are known in a few taxa of Acanthaceae, these are not among the fossil palynomorphs reported in Table 1. Fossils constrained the most recent common ancestor (MRCA) of the lineage to which the fossil could be unambiguously attributed (Appendix 1). When more than one fossil was available for the same taxon

set (Table 1 and Appendix 1), the oldest was used for calibration.

Divergence Time Analyses and Impact of Different Calibration Constraints

To explore the impacts of implementing different fossil constraints and employing a maximum age prior, we conducted four divergence time analyses: Analyses 1A and 1B (without and with a maximum age prior enforced), and Analyses 2A and 2B (without and with a maximum age prior enforced). For Analysis 1, we utilized both medium and high utility fossils (i.e., 3s, 4s and 5s) for a total of eight Acanthaceae calibration points after removal of younger fossils that date the same taxon set (see above and Appendix 1). For Analysis 2, we utilized only the highest utility fossils (i.e., 5s; again, only the oldest fossil date for a given taxon set used) for a total of four Acanthaceae calibration points. In both analyses, we also included one additional taxon set, comprising all Acanthaceae s.l., which we constrained to monophyly. In total, Analysis 1 contained nine taxon sets (Table 2) and Analysis 2 contained five taxon sets (Table 3). The maximum age prior used for Analyses 1B and 2B (i.e., the root of the tree) was modeled as a 125 Ma uniform prior (upper bound: 126.0 Ma; lower bound: 124.0 Ma), which is the age of the oldest generally accepted tricolpate fossil pollen of Eudicots (Sun et al. 2011). Eudicot pollen grains are unambiguously synapomorphic, readily fossilized and richly preserved in the fossil record. We chose this extremely conservative maximum prior in order to minimize the possibility that our basalmost divergence event would exceed the age estimate for this far more inclusive clade of angiosperms (Yang and Rannala 2006)

TABLE 2. Fossil constraints and priors used in Analysis 1

Taxon set	Fossil	Taxa constrained	Age	Zero offset	Log (SD)	Mean	5% quantile	95% quantile
TS 1	N/A	All Acanthaceae monophyletic	None designated (used tree prior)	N/A	N/A	N/A	N/A	N/A
TS 2	#1	<i>Avicennia</i> (3 species)	Mid Bartonian (~39.4–38.3 Ma)	38.3	0.6	0.5	38.46	39.42
TS 3	#11	<i>Acanthopsis</i> + <i>Acanthus</i>	Early–Mid Oligocene (~33.7–28.8 Ma)	28.8	1.1	1.5	28.9	33.8
TS 4	#15	Barlerieae (6 taxa)	Miocene (~23.8–5.3 Ma)	5.3	1.1	5.5	5.8	23.6
TS 5	#20	Justicieae (10 taxa)	Lower Miocene (~23.8–14.6 Ma)	14.6	1.3	2.5	14.7	23.7
TS 6	#32	NW Justicieae (3 taxa)	Lower Miocene (~23.8–14.6 Ma)	14.6	1.3	2.5	14.7	23.7
TS 7	#36	Pseudocolpate Ruellieae (6 taxa)	Upper Miocene (~14.55–5.3 Ma)	5.3	1.4	2.5	5.4	14.7
TS 8	#51	Petalidiinae + Mimulopsinae (3 taxa)	Mio-Pliocene (~23.8–1.8 Ma)	1.8	1.3	6	2.1	23.7
TS 9	#43	<i>Sanchezia</i> + <i>Strobilanthes</i>	12–10 Ma	10	0.5	1	10.4	12.0
[TS 10]	Tri-aperturate pollen	All taxa	127 Ma (max age, eudicots)	N/A	N/A	N/A	N/A	N/A

Note: Taxa constrained refers to crown groups. Fossil # as in Table 1.

TABLE 3. Fossil constraints and priors used in Analysis 2

Taxon set	Fossil	Taxa constrained	Age	Zero offset	Log (SD)	Mean	5% quantile	95% quantile
TS 1	N/A	All Acanthaceae monophyletic	None designated (used tree prior)	N/A	N/A	N/A	N/A	N/A
TS 2	#15	Barlerieae (6 taxa)	Miocene (~23.8–5.3 Ma)	5.3	1.1	5.5	5.8	23.6
TS 3	#51	NW pseudocolpate Ruellieae (3 taxa)	Mio-Pliocene (~23.8–1.8 Ma)	1.8	1.3	6	2.1	23.7
TS 4	#43	<i>Sanchezia</i> + <i>Strobilanthes</i>	10–12 Ma	10	0.5	1	10.4	12.0
TS 5	#32	<i>Dicliptera</i> + <i>Rhinacanthus</i> + <i>Justicia caudata</i>	Mid Miocene (~17.6–11.5 Ma)	11.5	1.5	1.6	11.5	17.6
[TS 6]	Tri-aperture pollen	All taxa	127 Ma (max age, eudicots)	N/A	N/A	N/A	N/A	N/A

Notes: Taxa constrained refers to crown groups. Fossil # as in Table 1.

while also allowing temporal “space” for estimating clade ages that are older than our fossil constraints, given that these provide minimum age estimates. Finally, we explored the effects of employing a much younger maximum age prior—that of the crown of Lamiales as estimated by Bell et al. (2010). These authors used Bayesian relaxed clock methods and 36 calibration points to estimate divergence times across more than 500 taxa of angiosperms. For this analysis—Analysis 3—we modeled a maximum age prior as a uniform prior ~74 Ma (upper bound: 78.0 Ma; lower bound: 68.0 Ma), a secondary calibration reflecting the age of crown Lamiales recovered in Bell et al. (2010).

We used BEAST v1.6.2 (Drummond and Rambaut 2007) to simultaneously estimate phylogeny and divergence times within Acanthaceae. BEAST was chosen over alternatives primarily because fossil calibrations can be input as probability distributions. Nucleotide substitution and clock models were set to unlinked across the seven data partitions, and different models of sequence evolution were applied to the seven partitions following results from Modeltest (Posada and Crandall 1998) hLRTs: ITS+5.8S: TrN+G, Nst=3; *psbA-trnH*: TVM+G, Nst=5; *rps16*: TVM+G, Nst=5; *trnG-trnR*: TVM+G, Nst=5; *trnG-trnS*: HKY+G, Nst=2; *trnL-trnF*: TVM+G, Nst=5; *trnT-trnL*: TVM+G, Nst=5; base frequencies were estimated for all loci. Rate heterogeneity across branches was permitted via implementation of a relaxed clock model (Drummond et al. 2006), and the uncorrelated lognormal distribution (UCLD) was selected because previous simulation study has demonstrated the superior performance of this distribution over others (Drummond et al. 2006). We used a uniform prior for UCLD means for each data partition, with an initial value of 1.0, an upper value of 100 and a lower value of 0.0 (default standard deviations [SDs] were utilized). A Yule Process speciation model was specified for the tree prior (Yule 1925; Gernhard 2008), and a random starting tree was used.

Lognormal priors were used for all Acanthaceae fossil calibrations (Drummond et al. 2006; Ho and Phillips 2009). A minimum age for each fossil was used as the

zero offset. Mean and SDs for each calibration were set such that 95% of the age distribution fell within the geological time period of the fossil in question (Tables 2 and 3). Given that fossils can provide only minimum ages of the clade to which they belong, one could consider extending the age distribution deeper into time than the lower bound of the empirically determined age of the fossil. However, there is no empirical basis for proceeding in this way in that we have no evidence-based lower or upper bounds other than those provided by the fossils. We therefore avoid speculation and use the only empirical data available to us: the estimated ages of the fossils. The maximum age priors enforced in Analyses 1B, 2B and 3 were implemented via adjustments to the “root height” parameter in BEAST. Chains were run for 50 million generations, logging every 500th generation.

We used Tracer v1.5 (Drummond and Rambaut 2007) to ensure that posterior distributions were sufficiently sampled. Using LogCombiner v1.7.3 (Drummond and Rambaut 2007), we thinned the number of trees to 10,000, including only post burn-in trees. Then, using TreeAnnotator v1.7.0 (Drummond and Rambaut 2007), we constructed maximum clade credibility trees from this thinned tree set and keeping target age heights.

Finally, we ran BEAST on the Analysis 1A data set sampling only from the prior, that is, without an alignment, to explore the influence of the remaining priors on the posterior distribution (Drummond et al. 2006).

Inferring Disjunction Events and Their Temporal Distributions

In instances where vicariance scenarios could clearly be rejected by our data, we inferred OW and NW disjunction events using the time-calibrated phylogeny herein presented in combination with extensive information on phylogenetic relationships within Acanthaceae from prior studies (see below). Importantly, we explicitly *did not* employ methods to reconstruct biogeographic events and dispersal timing

precisely because taxa herein sampled were intended to serve as phylogenetic placeholders for much more diverse evolutionary lineages (e.g., *Ruellia humilis* serves as a placeholder for a clade of some 300 species of NW *Ruellia*, which we know to be monophyletic based on prior study [Tripp 2007]), and reconstructing biogeography on the present taxon sampling would result in erroneous inferences in several cases. As one example, the African species *Dicliptera extenta* is here used as a placeholder for the monophyletic genus *Dicliptera* as a whole. Earlier and continuing work on tribe Justicieae (McDade et al. 2000b, in preparation), to which *Dicliptera* belongs, indicates that *Dicliptera* is monophyletic and includes one OW to NW dispersal event but none in the reverse direction (based on McDade et al. 2000b and additional unpublished data for a much richer taxonomic sampling of that genus). As such, we assumed a minimum of one OW to NW dispersal event in the present study. As a second example, in the present study we assumed an OW to NW dispersal event within the lineage that contains *Elytraria imbricata*. This information cannot be derived from the taxon sampling herein presented, but rather prior study of Nelsonioideae (the group to which *Elytraria* belongs) has demonstrated clearly that *Elytraria* is monophyletic and that there has been an OW to NW dispersal event within the genus (McDade et al. 2012). A third example is the OW to NW dispersal event inferred along the branch leading to *Barleria*. Although both taxa of *Barleria* herein sampled (*B. repens*, *B. lupulina*) are OW, we know that *Barleria oenotherioides*, the only NW species in a genus of >300 taxa, is strongly supported as nested within *Barleria* (S. Martín and Daniel, unpublished data). Future studies that conduct rigorous ancestral area analyses will require taxon sampling for lineages in the family specifically designed to address the questions at hand. The intention of the present study is to provide the tools to facilitate such studies.

We used knowledge from prior phylogenetic studies (Nelsonioideae: McDade et al. 2012; Thunbergioideae: Borg et al. 2008; *Avicennia*: Schwarzbach and McDade 2002; Acanthaceae: McDade et al. 2005; Barlerieae: McDade et al. 2008; Martín-Bravo and Daniel, unpublished data; Justicieae: McDade et al. 2000b; Kiel et al. 2006; Daniel et al. 2008; Ruellieae: Tripp 2007; Tripp and Manos 2008; Tripp et al. 2013) in combination with results from our time-calibrated phylogeny presented below to date disjunctions between OW and NW clades. None of the above-cited studies sampled exhaustively at the species level but all were designed to capture the taxonomic, morphological and biogeographic diversity encompassed by the lineage that was the focus of the study. Only among the basal lineages of Justicieae (termed the *Pseuderanthemum* clade by McDade et al. 2000b) is there any degree of uncertainty owing to taxonomic problems. However, no feasible pattern of OW/NW disjunctions would change the overall patterns reported here. This information was also used to discriminate among competing biogeographic hypotheses to explain present-day distributions, that is,

Gondwanan vicariance, Beringian land bridges, North American–European land bridges or long-distance dispersal.

Because results (see below) suggested that the timing of OW/NW disjunctions in Acanthaceae s.s. (>90% of the species diversity) was skewed toward the present, we tested whether disjunction events were more recent than expected by chance. To generate the null expectation, we randomly placed 11 disjunction events (i.e., equivalent to the observed number) onto 1000 trees sampled from the post burn-in posterior distribution of trees that resulted from Analysis 1A, thus taking phylogenetic uncertainty into account. All branches were equally likely to receive a disjunction event, but we weighted probability of receiving an event by the branch's length. We first pruned the two outgroups to generate a tree containing only the 70 ingroup taxa. For each of the 1000 sampled trees, we also determined the ages of the 11 observed disjunction events. For each tree, we determined the maximum age of the randomly placed disjunction event and assessed whether it was younger than the observed maximum age. The proportion of trees for which the random maximum age is younger than the observed maximum age gives a *P*-value for a conservative test of the null hypothesis that disjunction events are not skewed toward the present. We also compared the node age of observed and randomized disjunction events across the 1000 trees using a paired *t*-test. Analyses were conducted in R (source code available via Dryad doi: <http://dx.doi.org/10.5061/dryad.jm4d5>).

RESULTS

The seven genic regions contributed 9071 nt characters as follows: ITS: 884, psbA-trnH: 1449, rps16: 1133, trnGR: 1425, trnGS: 1399, trnLF: 1253 and trnTL: 1528. Significant non-clocklike molecular evolution was found among the 72 taxa ($H_0 = -\ln L = 67729.67$; $H_a = -\ln L = 66939.84$; $X^2 = 2579.66$, $P < 0.0001$, $df = 70$). Our alignment-free BEAST analysis (sampling only from the prior) resulted in extremely low posterior probabilities (e.g., there were no nodes in Acanthaceae s.l. with a posterior probability >0.11) and very unreasonable taxon assemblages, suggesting that the molecular matrix contained ample phylogenetic signal. In this analysis, posterior age distributions closely approximated ages of the priors.

Impact of Implementing Different Sets of Fossils and Maximum Age Priors

Our exploration of differences in estimates of clade age yielded three primary findings. First, clade age estimates were sensitive to different fossil inclusion sets. Analysis 1A (eight calibration points that span a longer temporal range) consistently yielded estimated clade ages older than Analysis 2A (four fossil constraints spanning a shorter and more recent temporal range), with exception

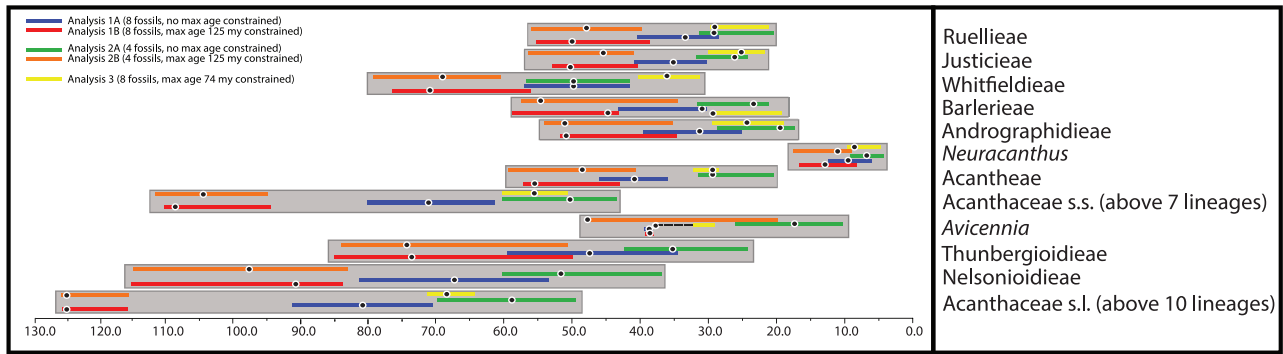


FIGURE 2. Colored bars indicate 95% HPD intervals for crown clade ages across five divergence time analyses conducted in this study: eight fossil constraints, without and with a maximum age of 125 Ma enforced (Analyses 1A and 1B), four fossil constraints, without and with a maximum age of 125 Ma enforced (Analyses 2A and 2B) and eight fossil constraints, with a maximum age of 74 Ma enforced (Analysis 3). Node ages depicted by filled black circles. In Analyses 1A, 1B and 3, both medium and high utility fossils were used as calibrations (i.e., ranks 3, 4, 5). In Analyses 2A and 2B, only highest utility fossils were used as calibrations (i.e., 5 only). Note that (1) implementation of 125 Ma maximum age prior results in substantially older clade ages (orange and red bars); (2) absent maximum age priors, an analysis that utilizes more fossils that span longer time periods results in older clade ages (purple bars); and (3) implementing a much younger maximum age prior (74 Ma) results in younger clade ages (yellow bars) and also forces non-monophyly of two of the basalmost lineages, Nelsonioideae and Thunbergioideae (hence lack of yellow bars for these). Acanthaceae s.s. encompass the bulk of species diversity (upper seven lineages in figure) in the family.

TABLE 4. Comparison of crown ages of major lineages of Acanthaceae (first 12 rows) and MRCA of other major splits (last 7 rows) across five different analyses: 8 fossil constraints, without and with a maximum age of 125 Ma enforced (Analyses 1A, 1B, respectively), 4 fossil constraints, without and with a maximum age of 125 Ma enforced (Analyses 2A, 2B, respectively) and 8 fossil constraints, with a maximum age of 74 Ma enforced (Analysis 3)

	1A	1B	2A	2B	3
Major lineages					
Acanthaceae s.l.	81.9 Ma [71.7–92.3]	125.8 Ma [116.2–125.9]	58.6 Ma [49.7–69.6]	124.3 Ma [115.8–125.8]	68.1 Ma [64.4–71.2]
Nelsonioideae	67.7 Ma [53.8–81.5]	90.8 Ma [83.7–115.3]	52.0 Ma [37.4–60.3]	97.7 Ma [83.4–115.1]	Not Monophyletic
<i>Avicennia</i>	38.7 Ma [38.4–39.3]	38.4 Ma [38.3–39.4]	17.2 Ma [10.2–26.5]	48.4 Ma [19.9–48.1]	38.5 Ma [28.8–32.1]
Thunbergioideae	47.2 Ma [34.5–59.5]	73.9 Ma [50.0–85.0]	35.7 Ma [24.6–42.6]	74.5 Ma [51.3–84.4]	Not Monophyletic
Acanthaceae s.s. (below seven lineages)	71.1 Ma [61.9–80.1]	108.5 Ma [94.1–111.0]	50.7 Ma [43.2–60.3]	104.3 Ma [95.0–112.0]	55.7 Ma [50.8–60.1]
Acantheae	41.2 Ma [36.1–46.4]	55.7 Ma [43.6–57.4]	29.2 Ma [20.6–32.0]	48.2 Ma [41.2–59.2]	29.2 Ma [28.8–32.1]
<i>Neuracanthus</i>	9.4 Ma [6.4–13.7]	13.1 Ma [8.8–16.9]	7.0 Ma [4.3–9.1]	11.4 Ma [8.9–17.5]	8.7 Ma [4.7–9.5]
Andrographidieae	31.9 Ma [25.1–39.0]	51.2 Ma [34.5–52.0]	19.5 Ma [17.3–28.9]	51.5 Ma [35.4–54.1]	24.4 Ma [18.9–29.3]
Barlerieae	31.5 Ma [30.3–43.6]	39.7 Ma [43.3–58.8]	23.7 Ma [21.6–32.2]	54.8 Ma [34.5–53.2]	29.9 Ma [19.1–29.9]
Whitfieldieae	49.7 Ma [42.1–57.2]	71.4 Ma [56.8–76.6]	49.7 Ma [42.0–57.1]	68.8 Ma [60.8–79.1]	36.1 Ma [31.5–40.4]
Ruellieae	33.9 Ma [28.0–40.7]	50.0 Ma [38.8–55.2]	29.3 Ma [20.4–31.1]	47.7 Ma [39.7–56.1]	29.8 Ma [21.8–29.9]
Justiceae	35.3 Ma [30.7–41.0]	50.4 Ma [40.3–53.8]	31.2 Ma [24.2–32.0]	45.8 Ma [41.3–56.3]	25.6 Ma [22.0–30.0]
Other major splits					
<i>Avicennia</i> /Thunbergioideae	70.9 Ma [61.4–80.7]	109.9 Ma [92.7–113.1]	49.6 Ma [42.4–60.2]	110.9 Ma [92.6–113.5]	Not Monophyletic
Acanthaceae/sister clade	71.1 Ma [61.9–80.1]	108.5 Ma [94.1–111.0]	50.7 Ma [43.2–60.3]	104.3 Ma [95.0–112.0]	55.7 Ma [50.8–60.0]
<i>Neuracanthus</i> /sister clade	58.4 Ma [51.5–66.5]	85.5 Ma [73.8–89.4]	43.8 Ma [35.5–48.5]	83.7 Ma [74.2–91.2]	46.5 Ma [41.3–49.5]
Andrographidieae/Barlerieae	46.7 Ma [39.9–53.7]	54.3 Ma [56.6–72.6]	36.0 Ma [28.4–40.6]	65.4 Ma [57.4–73.8]	34.9 Ma [31.5–40.4]
Whitfieldieae/sister clade	53.2 Ma [46.7–61.0]	78.1 Ma [65.9–81.8]	40.0 Ma [32.8–45.5]	72.9 Ma [66.9–83.8]	38.6 Ma [37.0–45.4]
Ruellieae/Justiceae	50.2 Ma [43.9–57.4]	71.7 Ma [62.4–77.6]	39.5 Ma [31.9–43.3]	68.3 Ma [62.4–79.4]	38.9 Ma [34.1–42.3]
Ruellieae+Justiceae/ sister clade	56.6 Ma [49.9–64.3]	80.3 Ma [71.3–86.4]	42.6 Ma [35.5–48.5]	79.1 Ma [72.6–89.1]	43.4 Ma [40.0–47.9]

Notes: Numbers in brackets refer to 95% HPD intervals of clade ages (note that because HPD intervals are calculated from all trees, node ages occasionally fall outside the interval, depending on what type of tree ages were summarized on; here, using target trees; see text).

of Whitfieldieae (Fig. 2; Table 4). Second, enforcing a maximum age prior of 125 Ma yielded dramatically older estimates of clade ages in both Analyses 1 and 2, that is, regardless of fossil inclusion set (Fig. 2; Table 4). For example, the age of crown Acanthaceae s.l. was 81.9 Ma in Analysis 1A versus 125.8 Ma in Analysis 1B and 58.6 Ma in Analysis 2A versus 124.3 Ma in Analysis 2B (see Table 4 for 95% HPDs). Third,

enforcing a maximum age prior of 74 Ma (Analysis 3) and using eight fossil calibration points (i.e., as in Analyses 1A and 1B) substantially altered the topology among basally branching lineages, with both Nelsonioideae and Thunbergioideae resolved as non-monophyletic (results not shown). Analysis 3 also yielded dates that were consistently younger than Analysis 1B (same fossil set but a maximum age prior of 125 Ma [vs. 74 Ma] in effect).

Analysis 1A is emphasized for the remainder of the results section because of the larger number of primary calibrations, because no secondary calibrations were invoked, and because maximum age constraints have been shown to be problematic in past studies (Hug and Roger 2007). See Discussion section for full explication.

Origin of Acanthaceae and Timing of OW/NW Disjunctions

Analysis 1A (more fossils, no max age) dates the node age of crown Acanthaceae s.l. to ~82 Ma (Fig. 3; see Table 4 for 95% HPD intervals; see Supplementary Fig. S1 [Supplementary Material; doi: <http://dx.doi.org/10.5061/dryad.jm4d5>] for a version

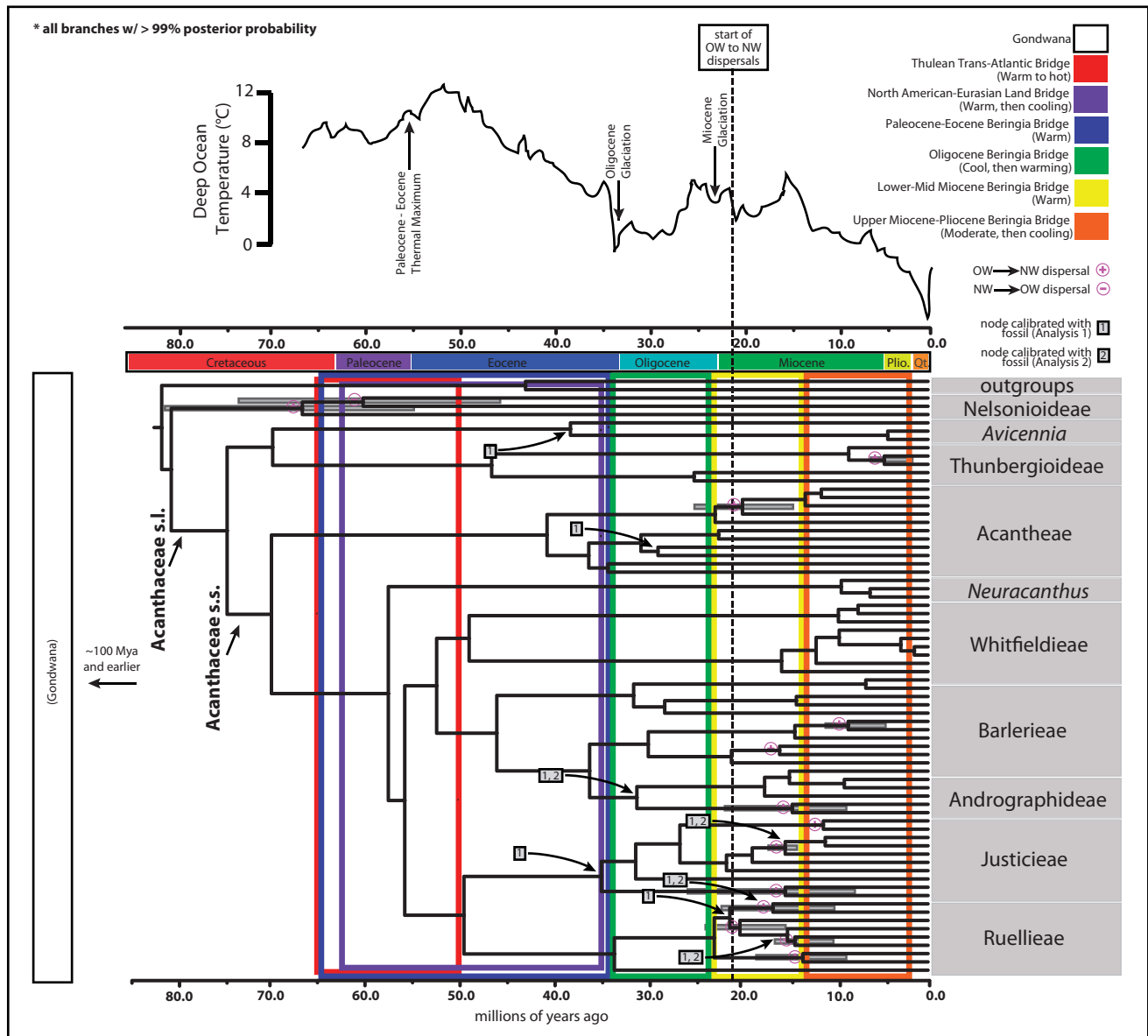


FIGURE 3. Best estimates of divergence times, major dispersal events within Acanthaceae based on BEAST Analyses 1A, and correlation with climatic and geological episodes in Earth history since late Cretaceous. Terminal taxa serve as phylogenetic place holders for more diverse clades for which we have more extensive phylogenetic information (see text: OW to NW dispersal events are inferred from other studies with more taxa per clade sampled). Circled plus signs denote an OW to NW dispersal event ($n=13$) and 95% HPD intervals are depicted on those branches with gray bars. Circled minus sign denotes the sole example of a NW to OW dispersal event ($n=1$; the lineage inclusive of *Elytraria* in Nelsonioideae). Dashed line represents the onset of OW to NW dispersals ($n=11$) in Acanthaceae s.s.; all occurred within the last ~20 Ma (significantly skewed toward the present) despite the fact that the lineage to which they belong is over three times as old. Small gray boxes with numerals identify nodes calibrated with fossils in Analysis 1 only, Analysis 2 only, or in both analyses (Tables 2 and 3). Major clades of Acanthaceae s.l. labeled at far right; Acanthaceae s.s. encompass Acantheae through Ruellieae in this figure. Temporal range of Gondwana and Atlantic and Pacific land bridges indicated by colored boxes overlaying phylogeny (key in upper right). Reconstruction of deep ocean temperatures (as a proxy for global temperature) is derived from oxygen isotopes corrected for variation in global ice volume (from Working Group I, 2007 Intergovernmental Panel for Climate Change report; see Fig. 6.1 therein). Approximate delimitation of climatic events presented is based on work of other authors (Wolfe 1975; Nilsen 1978; Mathews 1979; McKenna 1983; Tiffney 1985; see Burbrink and Lawson 2007 for partial summary). All branches have >99% posterior probabilities.

of Fig. 3 with species names provided instead of clade names). Early diverging lineages in the family such as Nelsonioideae, *Avicennia* and Thunbergioideae began to diversify between ~68 Ma and 39 Ma (crown ages; Fig. 3; 95% HPDs provided in Table 4). Acanthaceae s.s. (i.e., the lineage comprising >90% of species diversity) began to diversify ~71 Ma, shortly after crown Acanthaceae s.l. (Fig. 3; Table 4); major clades within Acanthaceae s.s. except for *Neuracanthus* diversified beginning ~50–32 Ma (crown ages).

Based on phylogenetic knowledge of lineages within the family (e.g., McDade et al. 2000a; McDade et al. 2000b; Schwarzbach and McDade 2002; McDade et al. 2005; Kiel et al. 2006; Tripp 2007; Borg et al. 2008; Daniel et al. 2008; McDade et al. 2008; McDade et al. 2012; Tripp et al. 2013) and in combination with data from this study, we infer (but do not explicitly optimize for reasons stated in the methods) a minimum of 16 disjunction events in Acanthaceae s.l. between the Eastern and Western Hemispheres. Thirteen of these (i.e., all events in Acanthaceae s.s. plus two others including Acanthaceae s.l.) were polarized in the OW to NW direction, one (i.e., in *Staurogyne*, Nelsonioideae, Acanthaceae s.l.) was in the NW to OW direction, and two (i.e., *Avicennia*, *Nelsonia*, both Acanthaceae s.l.) cannot be polarized owing to the widespread distributions of species (Fig. 3; Table 5). Additionally, Figure 3 and Table 5 show a skewed temporal distribution of ages of the 11 OW to NW disjunction events within Acanthaceae s.s., with all having occurred within the last 20.4 Ma even though Acanthaceae s.s. have been in existence for more than three times as long (Table 5). Upper limits of 95% HPD intervals were above 20.4 Ma for 5 of these 11 events, but only marginally so (i.e., with none extending beyond ~26 Ma; Fig. 3). Results from our simulation study of disjunction events through time rejected the null hypothesis ($P=0.009$ for maximum age test; $P=2.2 \times 10^{-16}$ for paired t -test of node ages), indicating that the observed distribution is significantly skewed toward more recent times than expected at random. In addition, this result is robust to uncertainty in phylogenetic topology and clade ages.

DISCUSSION

In addition to a general scarcity of fossils recovered from tropical latitudes, soft-wooded and herbaceous plant families are much more poorly represented in the fossil record than are woody ones owing to paucity of lignified tissue. In this study, we identified 51 reports of fossil Acanthaceae (and accepted all but one as representative of Acanthaceae, with varying degrees of confidence as reflected in Table 1) from a family of plants that, for the most part, is both tropical and not robustly woody. This stands in striking contrast to earlier statements that only a few fossils are known within Lamiales (23,000 + species) as a whole (e.g., Schäferhoff et al. 2010).

The utility of pollen in paleobotanical studies (Graham 2010) is largely attributable to three factors: its abundance (most seed plants produce copious pollen), its excellent fossilization potential (outer walls of pollen grains contain sporopollenin, a highly polymerized, lipid-rich molecule that is resistant to physical and chemical decomposition; Stanley and Liskens 1974; Zavada 2007) and its taxonomic utility, which varies among lineages. Bremekamp 1938, p. 135) acknowledged the taxonomic utility of pollen diversity in Acanthaceae in diagnosing not only tribes but “very often genera and species as well.” Our synthesis of a substantial number of fossil specimen reports from a large, tropical plant family in which pollen form is highly informative contributes important inferences about divergence times of this diverse and widespread family of plants and informs evolutionary histories of other tropical families, such as many in Lamiales (Olmstead and Tank, in preparation [personal communication]).

Impact of Different Fossil and Maximum Age Priors

Our results, particularly three notable patterns, demonstrate that different calibration regimes can substantially impact clade age estimates, as numerous other studies have shown (e.g., Bell et al. 2005; Hug and Roger 2007; Sauquet et al. 2012). First, clade age estimates were sensitive to different fossil inclusion sets: analyses that utilized more calibration points spanning a longer and deeper temporal range yielded older clade ages than analyses that utilized fewer calibration points that spanned a shorter, more recent temporal range (contrast Analyses 1A and 2A; Fig. 2). We attribute the much younger ages resulting from Analysis 2A to removal of our two oldest fossil calibrations (Fossil #1 [28.8 Ma] and Fossil #11 [38.3 Ma]). Whether by chance or because older fossils are rarer or may be inherently more likely to be judged less reliable, these were two of the rank “4” fossils that were excluded from Analysis 2. Second, enforcing a maximum age prior of 125 Ma yielded substantially older estimates of clade ages, regardless of fossil inclusion set (i.e., in both Analyses 1 and 2; Fig. 2). Notably, this impact is even greater than that of using different fossil sets (described above). Third, enforcing what we regarded to be a more reasonable maximum age prior of 74 Ma (rather than 125 Ma, above) yielded the anticipated result of consistently younger clade ages (Table 4; Fig. 3). Unexpectedly, it also resulted in non-monophyly of two of the basalmost lineages, Nelsonioideae and Thunbergioideae; these clades have been recovered as monophyletic in all phylogenetic analyses to date, regardless of taxon sampling schemes and molecular data sets employed (e.g., McDade et al. 2008; McDade et al. 2012).

The maximum age prior used for Analyses 1B and 2B was that for Eudicots as a whole although the vast majority of lineages of Eudicots are not represented in our analyses and Lamiales are relatively distally branching within Eudicots. We argue that our results

TABLE 5. Fourteen disjunction events in Acanthaceae s.l. inferred in the present study (Fig. 3), based on present taxon sampling in combination with previously published phylogenetic work (note that two additional disjunctions—one in *Nelsonia* and one in *Avicennia*—cannot be polarized owing to taxonomic uncertainty or pantropical distribution, respectively; these are not included in the table).

Lineage inclusive of / maximum age of dispersal	Maximum age of dispersal based on Analyses 1B, 2A, 2B and 3	Direction (reference)	Gondwana (≥ 96 Ma)	Thulean Trans-Atlantic bridge (~50–65 Ma)	North American–Eurasian land bridge (~35–63 Ma)	Paleocene–Eocene Beringia bridge (~34–65 Ma)	Oligocene Beringia bridge (~24–33 Ma)	Lower-Mid Miocene Beringia bridge (~14–23 Ma)	Upper Miocene–Pliocene Beringia (~3–13 Ma)
<i>Elytraria</i> 67.7 [53.8–81.5]	90.8 Ma 52.0 Ma 97.7 Ma Not monophy	OW to NW (McDade et al. 2012)	Yes [Yes]	Yes [No]	Yes [No]	Yes [No]	Yes [Yes]	Yes [Yes]	Yes [Yes]
<i>Staurogynne</i> 61.1 [46.4–74.8]	82.7 Ma 49.1 Ma 83.3 Ma Not monophy	NW to OW (McDade et al. 2012)	Yes [Yes]	No [No]	No [No]	No [No]	Yes [Yes]	Yes [Yes]	Yes [Yes]
<i>Mendoncia glabra</i> 4.7 [2.2–7.6]	5.7 Ma 2.5 Ma 8.6 Ma 2.7 Ma	OW to NW (Borg et al. 2008)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	No [No]
<i>Stenandrium pilosulum</i> 20.1 [15.0–25.4]	25.7 Ma 12.1 Ma 23.0 Ma 10.1 Ma	OW to NW (McDade et al. 2005)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [No]	No [No]	Yes [Yes]
<i>Barleria</i> 14.7 [8.9–21.5]	18.0 Ma 14.0 Ma 29.3 Ma 9.0 Ma	OW to NW (Bravo and Daniel, unpublished ms)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	No [No]	Yes [No]
<i>Lepidagathis alopecuroidea</i> and <i>Acanthura</i> 16.1 [10.3–21.6]	25.2 Ma 10.7 Ma 25.2 Ma 8.6 Ma	OW to NW (McDade et al. 2008)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	No [No]	Yes [No]
<i>Lophostachys</i> 8.6 [5.2–12.6]	13.9 Ma 5.7 Ma 14.0 Ma 5.0 Ma	OW to NW (McDade et al. 2008)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	No [No]
<i>Odontonema</i> 15.4 [8.0–26.5]	22.9 Ma 7.0 Ma 30.9 Ma 7.6 Ma	OW to NW (McDade et al. 2000b)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [No]	No [No]	Yes [No]
<i>Anisacanthus</i> 11.3 [7.1–15.8]	14.3 Ma 5.8 Ma 18.3 Ma 8.4 Ma	OW to NW (Daniel et al. 2008)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [No]	No [No]
<i>Justicia caudata</i> 15.4 [14.7–17.2]	20.5 Ma 15.6 Ma 17.1 Ma 8.7 Ma	OW to NW (McDade et al. 2000b)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	No [No]	Yes [No]
<i>Sanchezia</i> 16.8 [11.1–22.3]	24.5 Ma 12.0 Ma 22.3 Ma 11.6 Ma	OW to NW (Tripp et al. 2013)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	No [No]	Yes [No]
<i>Dyschoriste</i> 14.4 [11.2–17.7]	25.1 Ma 10.5 Ma 22.8 Ma 9.4 Ma	OW to NW (Tripp et al. 2013)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	No [No]	Yes [No]
<i>Hygrophila</i> 20.4 [16.4–24.2]	32.3 Ma 11.3 Ma 23.2 Ma 15.4 Ma	OW to NW (Tripp et al. 2013)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [No]	No [No]	Yes [Yes]
<i>Ruellia</i> 13.5 [9.3–18.3]	21.2 Ma 9.6 Ma 11.1 Ma 9.4 Ma	OW to NW (Tripp 2007; Tripp and McDade 2014)	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [Yes]	Yes [No]	Yes [No]

Notes: Maximum ages based on node ages derived from Analysis 1A followed by 95% HPD (Column 1) or node ages derived from Analyses 1B, 2A, 2B and 3 (Column 2). The 14 disjunctions for which we infer direction of dispersal are all OW to NW except for one (see Discussion section), and those that occurred ≤ 20.4 Ma are significantly non-random through time (see text). Entries in **bold** refer to the 11 OW to NW dispersals in Acanthaceae s.s., a clade that comprises $>90\%$ of species diversity in the family. Columns 4–10 are based on Analysis 1A and show whether Gondwana, the Thulean Land Bridge, North American–Eurasian Land Bridge, or one of four Beringian Land Bridges can be rejected by mean age [or by 95% HPD interval, in brackets afterwards] of clade as hypotheses to explain the clade's OW/NW distribution. Data presented in this study reject Gondwana, Thulean and North American–Eurasian Land Bridges as explanations for OW/NW disjunctions in Acanthaceae s.s. Beringian Land Bridge scenarios are often rejected by node ages, but not always by age HPDs. These results favor multiple long-distance dispersals as the primary explanatory process for modern Acanthaceae distributions (see Discussion section). See Figure 3 caption for land bridge references. Note that some land bridges were contemporaneous.

reveal that use of this 125 Ma age prior is inappropriate because it pulls clade ages “back” toward the tree root age, effectively down-weighting the impact of the fossils themselves. Thus, a consequence of implementation of a maximum age prior that is too old includes unreasonably deep divergence times (Hug and Roger 2007). We attempted to rectify this problem by employing what we regarded as a more reasonable maximum age prior of 74 Ma (based secondarily on the age of crown Lamiales estimated by Bell et al. 2010), but this analysis returned unexpected non-monophyly of two of the basalmost lineages of Acanthaceae (Nelsonioideae and Thunbergioideae). Invoking a maximum age prior that is too young apparently forced estimated ages to be younger than those optimized using data on fossil calibration points, branch lengths and phylogenetic relationships in concert (Yang and Rannala 2006; Ho and Phillips 2009). In effect, Analysis 3 forced too much evolutionary change into too little time, and the non-monophyly of Nelsonioideae and Thunbergioideae must in part be attributed to their long-branch lengths (typical of early diverging lineages that are still extant) and the very deep bifurcations within those lineages (Fig. 3).

These results corroborate prior work that has documented similar dramatic age differences when using or not using maximum age priors (e.g., Hug and Roger 2007) and suggest that use of such priors requires careful consideration and justification. Ho and Phillips (2009, p. 370) described the difficulty in reaching a compromise between “choosing a young bound that might exclude the true divergence time, and choosing an old bound that is too uninformative.” Ksepka et al. (2011, p. 3) similarly noted difficulties with specifying maximum ages and concluded that, ultimately, whether or not to do so should be optional, balancing “the need for palaeontologists to inform maximum ages against the difficulties in justifying maxima in cases where the fossil record is poor or phylogenetic uncertainty is high.” These phenomena—how BEAST handles maximum ages and fossil sets that differ markedly in age—continue to merit further examination, ideally in a methodological study that systematically explores the impact of these and other factors.

Marshall (1997, 2008, 2010) proposed objective methods for establishing maximum ages or confidence intervals on stratigraphic ranges (also see Dornburg et al. 2011); other authors have pointed to the impact of biases in preservation through time on these methods (Lu et al. 2006). We were unable to implement these methods owing to an insufficient number and distribution of fossils for any given lineage and a lack of sufficient fossils from close relatives. Indeed, the methods of Marshall (2010) are highly attractive additions to the divergence time analytical toolbox but unfortunately can be readily implemented only by systematists who work on the most fossil-rich lineages.

We emphasize results from Analysis 1, specifically 1A, for several reasons. (1) Analysis 1A is based on more calibrations than Analysis 2 (i.e., eight vs. four). (2) The subset of fossils used for Analysis 2 omitted

two of our oldest fossils (*Avicennia*: Fossil #1, ~38–39 Ma and *Acanthus*: Fossil #11, ~29–34 Ma), which would seem to be critical for accurately estimating ages of deeper branches in the phylogeny. (3) Analysis 1A, unlike Analyses 1B, 2B and 3, does not invoke calibrations far removed from the ingroup (i.e., the eudicot maximum age constraint [Analysis 2] or secondary calibrations for crown age of Lamiales based on Bell et al. (2010) [Analysis 3]), which can lead to biased age estimates (Sauquet et al. 2012). (4) Prior studies have highlighted problems with maximum age calibrations (discussed above). Although not all fossils in Analysis 1 were ranked the highest (as in Analysis 2), all were judged to be of high quality and sufficiently reliable for use as calibration priors. We thus conclude that results from Analysis 1, specifically 1A, are most likely to reflect true clade ages of Acanthaceae.

Divergence Time Estimations of Major Clades of Acanthaceae

Results from Analysis 1A (from fossils judged to be medium to high quality) indicate that crown groups of major clades (i.e., for the most part, tribes) within Acanthaceae s.l. began diversifying over an ~18 myr period (crown Whitfieldae [oldest] = 49.7 Ma and crown Barlerieae [youngest] = 31.5 Ma; Table 4). One exception is the taxonomically modestly sized and phylogenetically poorly resolved genus *Neuracanthus* which, based on this taxon sampling, began diversifying within the last ~9 Ma despite having originated contemporaneously with other major lineages of Acanthaceae (Fig. 3). We caution, however, that this result may be due to inadequate taxon sampling within *Neuracanthus* (see Methods section) and that addition of more taxa may push the crown age of *Neuracanthus* back in time. Within Acanthaceae s.s., our divergence time analysis dated crown groups Justiciaeae and Ruellieae—the two largest tribes and sister clades—to ~35 Ma and 34 Ma, respectively. Except in the case of *Neuracanthus* as just discussed, it is very unlikely that denser taxon sampling will substantially alter age estimates herein provided because of our taxon sampling strategy (see Methods section; additional discussion below).

Earlier studies included Acanthaceae in age estimates of Asterids (Bremer et al. 2004) and in an angiosperm-wide analysis (Bell et al. 2010, online appendix S23). Both studies included taxa that nearly spanned basal dichotomies in the family s.l. (i.e., *Avicennia* or *Thunbergia* plus one member of Acanthaceae s.s.). In these studies, crown group Acanthaceae s.l. were dated to be 54 Ma (Bremer et al. 2004) or 41 Ma (Bell et al. 2010; this age taken from their analysis that implemented lognormal priors). In our study, crown group Acanthaceae s.l. were dated to ~82 Ma, confirming prior findings that the use of different taxon sampling and different fossil calibration priors can substantially affect clade ages (Sauquet et al. 2012). We emphasize clade age results from our study because it uses direct fossil evidence from the family as well as a phylogenetically informed taxon sample.

Species sampled for the present study represent a very small fraction of the total diversity of Acanthaceae, which raises the question as to whether and how our dates and biogeographic inferences would differ if based on alternative taxon samples. Importantly, our taxon sampling strategy was carefully devised for this study. (1) Within each major clade (i.e., tribe, for the most part), we were careful to ensure that the basalmost node of the lineage was sampled. This was made possible by prior phylogenetic research in numerous phylogenetic “neighborhoods” within the family, with taxon sampling in each case designed to represent taxonomic, morphological and biogeographic diversity (citations throughout). (2) We explicitly selected taxa for analysis that we knew to represent the rich biogeographic history of Acanthaceae. Indeed, the present study was in part motivated by exciting prospects of using rigorously vetted fossils in finer scale analyses of divergence and diversification among closely related species (Tripp and McDade 2014). One example is the Neotropical genus *Ruellia*, which exhibits numerous transitions in pollination systems and for which we have molecular data for over half of 300+ species (Tripp and Manos 2008; Tripp and McDade 2014). Each future fine-scale study will require its own, carefully devised taxon sample.

Discriminating among Biogeographical Hypotheses

Assuming that minimum age estimates based on fossils used in this study approximate true clade divergence times, vicariance associated with the break-up of Gondwanaland is too old for all lineages, including the basalmost lineages (Analysis 1A). Therefore, land bridge migrations or long-distance dispersals are favored scenarios to explain OW/NW disjunctions in Acanthaceae. Land bridges connecting the Eastern and Western Hemispheres within the last ~65 Ma were Beringian, with the exception of the Trans-Atlantic Thulean Land Bridge that was present around the Paleocene–Eocene Thermal Maximum (~65–50 Ma; Nilsen 1978) and a North American–European (i.e., Boreotropical) Land Bridge that was present from the Early Paleocene to the Late Eocene (~65–35 Ma; Wolfe 1975; Tiffney 1985; Lavin and Luckow 1993). Ample evidence suggests that multiple Beringian land bridges formed and submerged on several occasions within the last 65 Ma (summarized in Burbrink and Lawson 2007; Fig. 3).

Data we present cannot explicitly reject all hypotheses of land bridge migration in favor of long-distance dispersals (Table 5), in part because fossils provide minimum estimates of ages (Heads 2011). Notably, the two most recent Beringian bridges (Lower-Mid Miocene and Upper Miocene–Pliocene; Fig. 3) coincided with many of the estimated ages of the migration/dispersal events in Acanthaceae s.s. However, four sources of evidence strongly favor long-distance dispersal over migration via these land bridges. (1) Our results

indicate that all 11 of the OW to NW disjunction events in Acanthaceae s.s. occurred within the last ~20 Ma, a period in which global temperatures were cooler to slightly warmer than present at best. We would expect that, for plants from tropical lineages to successfully migrate via Beringia, global temperatures would have to have been substantially warmer than at present (cf. the Paleocene–Eocene Maximum; Fig. 3). (2) All of the OW/NW disjunctions in Acanthaceae s.s. are between groups that have their modern distributions in Africa and the Neotropics rather than between Asia (or Europe) and the Neotropics. (3) There are species-rich lineages in Asia (e.g., *Strobilanthes*, >400 species; Andrographideae, ~90 species) that are completely unrepresented in the NW; if Beringia were a primary mode of land bridge migration, one might expect these species-rich Asian lineages to be present in the NW. (4) If lineages of Acanthaceae commonly utilized Beringian land bridges, they might be expected to have left behind fossils. Table 1 reveals two instances of medium to high utility fossils that were recovered in extratropical regions: an *Acanthus* fossil from the UK (~34–29 Ma old) and an *Avicennia* fossil from Spain (~39–38 Ma). However, there are no species of *Acanthus* native to the NW, and biogeographical inferences based on *Avicennia* are difficult owing to its present-day pantropical and subtropical distribution. Thus, whereas many of our results are consistent with both land bridge migration and long-distance dispersal scenarios, climate data, present-day distributions, a lack of Asian–NW sister groups and a paucity of extratropical fossils favor long-distance dispersal as the primary mode of the multiple lineage translocations herein described. Seeds of Acanthaceae are small in size (many <5 mm in diameter), perhaps making them transportable by vectors including wind or animals. Additionally, numerous species have seeds covered by hygroscopic trichomes that become mucilaginous when wet, perhaps facilitating secondary long-distance dispersals via attachment to animals.

Does Uncertainty in Timing Affect Biogeographical Conclusions?

We estimated divergence times using five different constraint scenarios to explore whether fossil inclusion sets and/or maximum age priors affected estimated clade ages to an extent that would impact our overarching biogeographical conclusions (cf. Smedmark et al. 2010). Under all five scenarios, OW/NW disjunction ages were consistently too young to support a Gondwanan vicariance hypothesis (Table 5). Additionally, none of the OW/NW disjunctions under any of the five scenarios was older than ~32 Ma (Oligocene), and the vast majority of disjunction events independent of analysis type was still <20 Ma (Table 5). The results of Analyses 1B and 2B yielded dates for some disjunctions that were sufficiently

long enough ago to have occurred when the Earth was warmer than present (Fig. 3). However, if these land bridges were modes of dispersal, no fossils yet discovered were left behind (see also Discussion section above). Thus, although estimated clade ages vary among the five analyses, and sometimes substantially, our biogeographic conclusions—that long-distance dispersals likely primarily explain extant Acanthaceae distributions—are robust. Long-distance dispersal is increasingly recognized as a primary mechanism in the biogeographic histories of many angiosperm families (Christenhusz and Chase 2013).

Biases in Directionality and Timing of OW/NW Dispersals

Based on taxon sampling in the present study together with knowledge of clade constituents derived from our earlier and ongoing research, we have documented a minimum of 13 dispersal events that were in the OW to NW direction (Table 5 and references therein). Remarkably, we have documented only one event that was in the reverse direction. We are unable to derive any conclusion from two additional events, in *Avicennia* and *Nelsonia*, because species are nearly cosmopolitan in distribution.

Because >90% of 4000+ species in Acanthaceae are members of Acanthaceae s.s., we gave special attention to the 11 dispersal events within this clade (Fig. 3). Acanthaceae s.s. includes all lineages except the basal members Nelsonioideae (~175 spp.; McDade et al. 2012), *Avicennia* (~8 spp., Schwarzbach and McDade 2002) and Thunbergioideae (~169 spp.; Borg et al. 2008). In a theoretical framework, the number of dispersal events across a phylogeny should be positively correlated to time or, in the case of an ultrametric tree, to branch lengths (i.e., time should be positively correlated to dispersal opportunity). Intriguingly, data from Analysis 1A and our simulation study demonstrate that the 11 OW to NW dispersal events in Acanthaceae s.s. were instead significantly skewed toward the present, with all events occurring within the last ~20 Ma even though this lineage has been in existence over three times longer (Fig. 3; Table 4). These results are intriguing because, barring changes in geographic proximity or in dispersal mechanisms, long-distance dispersals should be random through time. We are unaware of hypotheses to explain this bias in the biogeographic history of Acanthaceae, but this pattern is repeated in other plant groups (e.g., Chrysobalanaceae; Bardon et al. 2013) and is ripe for future investigation.

Jurassic and Triassic Fossil Reports

In 1992, Cornet and Habib reported a fossil palynomorph from the Upper Jurassic of France (Oxfordian, ~155–161 Ma; as *Multimarginites* sp.), with surface traits remarkably consistent with Trichantherinae in Ruellieae (a tribe that arguably possesses the most distinctive of all pollen types

found among Acanthaceae; Fig. 1e,f). Traits aligning this fossil palynomorph with present-day pollen of Trichantherinae (sensu Tripp et al. 2013) include: prolate, bi-aperturate grains with numerous bands of pseudocolpi that are oriented 90° from each other on opposing faces (Fig. 1e,f). Four years earlier, Pocock and Vasanthi (1988) reported a different palynomorph from an Upper Triassic (Carnian, ~217–229 Ma; as *Cornetipollis reticulata*) formation in Arizona that they likewise attributed to Trichantherinae. Grains were again remarkably consistent with modern-day Trichantherinae pollen, but lacked the two apertures. Because these ages vastly predate any estimates of the origin of angiosperms, we ranked these reports “2” and did not use them in our analyses. However, we remain intrigued as to the history of these peculiar fossils. Were these fossils indeed representative of stem Trichantherinae (especially *Multimarginates*, which essentially matches the surficial morphology of modern Trichantherinae in every regard), and age estimates reported in (Cornet and Habib 1992) and Pocock and Vasanthi (1988) were drastically wrong? Or were these fossils dated accurately and, remarkably, this highly apomorphic fossil morphology in fact evolved early on in the pre-angiosperm fossil record? Given all evidence available to us at this time, we can only conclude that their ages are extreme outliers in the broader distribution of ages of our 51 fossil reports (Table 1) and indeed of all other fossils accepted by scientists as representative of angiosperms as a whole.

In a thoughtful but infrequently cited paper, Zavada (2007) provided thorough discussion of these early reports. We agree that these and other ancient fossils with angiosperm-like features amply merit continued study and evaluation. Zavada reviewed the palynological evidence for an origin of angiosperms long before the lower Cretaceous estimate that has been accepted in many studies based on reliable fossil evidence (e.g., see Magallón and Castillo 2009; Bell et al. 2010). He noted that there are numerous pre-Cretaceous (Triassic, Jurassic) fossil palynomorphs that unequivocally have angiospermous synapomorphies, and argued that these likely represent stem group lineages basal to extant angiosperms. These early “pre-angiosperms” would not have yet evolved the full suite of synapomorphies of flowering plants, and Zavada (2007) observed that similarly aged fossils of other organs with angiosperm traits have not been discovered. He also offered the possibility that palynological traits that we understand to be synapomorphic for angiosperms may have evolved in parallel in now extinct plants unrelated to angiosperms. Finally, Zavada (2007) suggested that during these early stages of angiosperm evolution, the pace of diversification might have been matched by that of extinction such that these early plants with angiospermous pollen grains did not dominate the Earth’s floras as have modern flowering plants and thus would not have been expected to leave many meso- or macrofossils.

CONCLUSIONS

Error associated with fossil-calibrated divergence time estimations generally falls into one of two categories: error associated with the fossils (e.g., age, identification, lack of sufficient or sufficiently informative fossils) and error associated with the estimations (e.g., phylogenetic uncertainty, improper calibration procedures, violation of assumptions, substitution rate variation; [Rutschmann et al. 2007](#)). These sources of error, and approaches to minimizing or dealing with them, have been discussed extensively elsewhere (see, e.g., [Gandolfo et al. 2008](#)). In this study, we attempted to minimize both sources of error by (1) utilizing an unexpectedly rich fossil history of the plant family Acanthaceae to provide multiple calibrations; and (2) exploring the effects of implementing maximum age priors in a framework that accounts for phylogenetic uncertainty. The end product is our best estimate of a time-calibrated phylogeny that provides a temporal context for major clade divergences in a diverse, widespread and ecologically important tropical family of angiosperms.

A robust, time-calibrated phylogeny of Acanthaceae opens numerous new avenues of research in Acanthaceae, particularly comparative diversification analyses in many lineages of interest. Within the family, several clade-specific studies (e.g., *Justicieae*: [McDade et al. 2000b](#); [Kiel et al. 2006](#); [Daniel et al. 2008](#); *Acantheae*: [McDade et al. 2005](#); *Ruellia*: [Tripp 2007](#); [Tripp and Manos 2008](#); “*BAWN*”: [McDade et al. 2008](#); *Ruellieae*: [Tripp et al. 2013](#)) have documented numerous lineages with distributions in both the OW and the NW. Within these lineages, it is often the case that NW clades are more species-rich than OW clades ([Tripp et al. 2013](#)). This calls for detailed examination of patterns within these lineages along with exploration of correlates of clade size asymmetries that may point to responsible processes (e.g., [Tripp and McDade 2014](#)). This study sets the stage for research that will take advantage of the species-richness, morphological disparity and broad geographic distribution of Acanthaceae to frame the biological features of diversification in the temporal and spatial context provided by the calibrated phylogeny.

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.jm4d5>.

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APPENDIX 1

In-depth explanation for rankings of all 51 fossil reports considered in this study, and description of synapomorphies (for fossils used) that guided our delimitation of Taxon Sets (see manuscript). Column 1: Taxon name refers to modern taxon. In instances where fossil was used in Analysis 1, 2 and/or 3 (i.e., those in **bold**), the identification provided for the fossil in the primary reference (Table 1) follows the modern taxon. Column 2: # refers to fossil # as in Table 1. Column 3: U = Fossil utility score as in Table 1. Column 4: For fossils used in analyses (i.e., **bolded**), explanation of phylogenetic and synapomorphic justification for placement of fossil constraints. Column 5: Justification for fossil utility scores presented here and in Table 1.

Taxon	#	U	Synapomorphy justification	Utility score justification
<i>Avicennia</i> (<i>Avicennia</i> lineage); Fossil Taxon: not named, but compared to modern taxa <i>Avicennia alba</i> and <i>Avicennia marina</i>	1	4	<i>Avicennia</i> are monophyletic (Schwarzbach and McDade 2002) and share unique pollen features: spheroidal and tricolporate with longitudinally elongated ora and reticulate surface ornamentation with broad muri and small lumina (Borg and Schonenburger 2011); these fossils share these traits and were used to constrain the MRCA of the three sampled spp. of <i>Avicennia</i> .	Ranked “4” because pollen cores from which fossils were sampled were from mangrove habitat; not given highest rank (i.e., “5”) because no image of fossil provided.

(continued)

Taxon	#	U	Synapomorphy justification	Utility score justification
<i>Avicennia</i> (<i>Avicennia</i> lineage)	2	1	Fossil not used in analyses.	Ranked "1" because (Burnham and Graham 1999) is a secondary report (i.e., based on an earlier report), because older fossils exist that calibrate the same nodes (i.e., MRCA of <i>Avicennia</i> , fossil #1), and because no image provided.
<i>Avicennia</i> (<i>Avicennia</i> lineage)	3	2	Fossil not used in analyses.	Ranked "2" owing to minimal discussion in Gruas-Cavagnetto et al. (1987, p. 226): "C'est à Valeron (Cabardès) et à Lagrasse (Corbières) que l'on trouve les associations les plus riches, notamment en éléments de végétation littorale (<i>Nypa</i> , <i>Avicennia</i>)..." and to lack of image.
<i>Avicennia</i> (<i>Avicennia</i> lineage)	4	2	Fossil not used in analyses.	Ranked "2" because (MacPhail et al. 1994) is a secondary report (i.e., based on an earlier report noted as unconfirmed); moreover, no image was provided.
<i>Mendoncia</i> (Thunbergioidieae)	5	1	Fossil not used in analyses.	Ranked "1" because (Burnham and Graham 1999) is a secondary report (i.e., is based on an earlier report), because it is extremely young (i.e., Quaternary), and because no image was provided.
Acanthaceae	6	2	Fossil not used in analyses.	Ranked "2" because (Hooghiemstra et al. 2006) assigned only to "Acanthaceae" and no images were provided.
Acanthaceae	7	2	Fossil not used in analyses.	Ranked "2" because (Hooghiemstra et al. 2006) assigned only to "Acanthaceae" and no images were provided.
Acanthaceae	8	2	Fossil not used in analyses.	Ranked "2" because (Hooghiemstra et al. 2006) assigned only to "Acanthaceae" and no images were provided.
Acantheae	9	2	Fossil not used in analyses.	(Medus 1975) provides images of colpate fossil pollen grains that may very well represent Acantheae, but we ranked this as "2" because we deemed it less useful than fossil #11, which is a seed macrofossil of similar age.
<i>Acanthus</i> (Acantheae)	10	4	Fossil not used in analyses.	Ranked "4" because (Morley's 1977) fossil reliably identified, but not used in our analyses because fossil #11 is older and constrains the same MRCA; moreover, no image was provided.
<i>Acanthus</i> (Acantheae); Fossil Taxon: <i>Acanthus rugatus</i> Reid and Chandler	11	4	Monophyly of <i>Acanthus</i> cannot be rejected by molecular data (McDade et al. 2005); plants produce seeds with distinctly rugose surface sculpturing (Tomlinson 1986) exactly like that of this fossil; thus, fossil used to constrain the MRCA of the lineage containing <i>Acanthus semii</i>.	Ranked "4" because (Reid and Chandler 1926) report provide an image of a macrofossil (seed) that we deem reliably assigned to <i>Acanthus</i>; not ranked "5" because seed sculpturing has not been exhaustively surveyed across tribe Acantheae.
<i>Acanthus</i> (Acantheae)	12	2	Fossil not used in analyses.	Ranked "2" because older and more reliable fossils exist with which to calibrate the MRCA of <i>Acanthus</i> (i.e., fossil #11) and because no image was provided.
<i>Blepharis</i> (Acantheae)	13	1	Fossil not used in analyses.	Ranked "1" because other reports of fossil Acantheae predate this extremely young fossil (i.e., Holocene) by ~20–30 myr; moreover, no image was provided.
<i>Blepharis</i> (Acantheae)	14	2	Fossil not used in analyses.	Ranked "2" because other reports of fossil Acantheae predate this extremely young fossil (i.e., Quaternary) by ~20–30 myr; image indicates that it is accurately identified.
<i>Hulemacanthus</i> (Barlerieae) Fossil Taxon: not identified	15	5	<i>Hulemacanthus</i> pollen is prolate, tricolporate with broadened colpi, and has coarsely reticulate exine sculpturing (Scotland and Vollesen 2000); we agree with (Raj 1961) assignment of fossil #15 to this genus. Phylogenetic placement of <i>Hulemacanthus</i> within Barlerieae remains unknown (McDade et al. 2008) thus we constrain the MRCA of all Barlerieae with this fossil.	Ranked "5" because of shared synapomorphic traits with extant plants. Kuyl et al. (1995) provided a series of plates of reliably dated but mostly unidentified fossils, including this one. (Raj 1961) discovered Kuyl's publication and attributed Kuyl's Plate 4, Figure 6 to the monotypic genus <i>Hulemacanthus</i>.

(continued)

Taxon	#	U	Synapomorphy justification	Utility score justification
<i>Lepidagathis</i> (Barlerieae)	16	2	Fossil not used in analyses.	Ranked "2" because we are uncertain that fossil can be reliably assigned to the clade containing <i>Lepidagathis</i> based on image provided; merits further study.
Whitfieldieae or Isoglossinae (Justicieae)	17	2	Fossil not used in analyses.	Ranked "2" because fossil pollen dated to the mid Cretaceous, ca. 125–99 mybp or substantially older than other reports of Acanthaceae. Merits further study as image indicates pollen similar to modern Whitfieldieae or Isoglossinae.
Justicieae	18	4	Fossil not used in analyses.	Ranked "4" because image provided appears to be correctly identified (not ranked 5 because of concern for possible contamination of its source, the Ziguinchor core); not used in analysis because fossil #20 is older and constrains the same MRCA.
Justicieae	19	4	Fossil not used in analyses.	Ranked "4" because image provided appears to be correctly identified (not ranked 5 because of concern for possible contamination of its source, the Ziguinchor core); not used in analysis fossil #20 is older and constrains the same MRCA (also).
Justicieae Fossil Taxon: <i>Areolipollis</i> <i>vespiformis</i> Clarke and Frederiksen <i>Dicliptera</i> (Justicieae)	20	4	This fossil is hexapseudocolpate, synapomorphic for Justicieae (McDade et al. 2000b); assigned to the MRCA of Justicieae.	Ranked as "4" because the image provided in (Beialy et al. 2005) is most likely of pollen of Justicieae; not ranked "5" because poor image quality precluded closer study.
<i>Dicliptera</i> (Justicieae)	21	2	Fossil not used in analyses.	Ranked "2" because, although an image of the fossil was provided and it appears to be correctly identified, other reports of fossil Justicieae significantly predate this extremely young fossil (i.e., Quaternary).
<i>Glossochilus</i> (Justicieae)	22	2	Fossil not used in analyses.	Ranked "2" because, although the image of the fossil palynomorph provided by (Scott 1982), Plate 4:K) is remarkably similar to modern <i>Glossochilus</i> (bicolporate, 4-pseudocolpate), a genus restricted to South Africa, where this fossil was recovered, other reports of fossil Justicieae predate this extremely young fossil (i.e., Quaternary).
<i>Hypoestes</i> (Acantheae)	23	1	Fossil not used in analyses.	Ranked "1" because other reports of fossil Justicieae predate this fossil (i.e., Pliocene); moreover, no image was provided.
OW <i>Justicia</i> (Justicieae)	24	2	Fossil not used in analyses.	Ranked "1" because other reports of fossil Justicieae predate this fossil (i.e., Pliocene); moreover, no image was provided.
<i>Metarungia</i> (Justicieae)	25	2	Fossil not used in analyses.	Ranked "2" because other reports of fossil Justicieae significantly predate this very young fossil (i.e., late Quaternary); moreover, the image provided cannot be placed confidently.
<i>Monechma</i> (Justicieae)	26	2	Fossil not used in analyses.	Ranked "2" because other reports of fossil Justicieae significantly predate this very young fossil (i.e., late Quaternary); moreover the image provided cannot be placed confidently.
NW <i>Justicia</i> (Justicieae)	27	1	Fossil not used in analyses.	Ranked "1" because (Burnham and Graham 1999) is a secondary report (i.e., based on an earlier report), because fossil is extremely young (i.e., Quaternary), and because no image was provided.
NW <i>Justicia</i> (Justicieae)	28	4	Fossil not used in analyses.	Ranked "4" because image provided in (Graham 1976) is an excellent match for <i>Justicia</i> (specifically NW <i>Justicia</i> , given location where palynomorph was recovered); not used in analysis because fossil #32 is older, ranked "5", and dates the same clade.
NW <i>Justicia</i> (Justicieae)	29	2	Fossil not used in analyses.	Ranked "3" because the image provided in (Germeraad et al. 1968) resembles <i>Justicia</i> (specifically NW <i>Justicia</i> , given location where palynomorph was recovered); however, fossil #32 is older and dates the same clade and so was used for analyses.

(continued)

Taxon	#	U	Synapomorphy justification	Utility score justification
NW <i>Justicia</i> (Justicieae)	30	2	Fossil not used in analyses.	Ranked as "2" because despite discussion of fossils, we have been unable to acquire images of this fossil.
NW <i>Justicia</i> (Justicieae)	31	1	Fossil not used in analyses.	Ranked "1" because of inadequate information; moreover, we are unable to access images from the publication.
NW Justicieae Fossil Taxon: <i>Areolipollis insularis</i> Mautino	32	5	This fossil is dicolporate with distinctive areoles that surround the germinal apertures, traits known only from NW Justicieae (Graham 1988).	Ranked "5" because image provided in (Mautino 2011) is an excellent match for <i>Justicia</i> (specifically NW <i>Justicia</i> , given verrucae that surround the apertures and location where palynomorph was recovered). Fossil not used in Analysis 1 because fossil #30 predates it. Used in Analysis 2 instead of fossil #29 because its stratigraphic range is narrower.
OW <i>Justicia</i> (Justicieae)	33	2	Fossil not used in analyses.	Ranked "2" because other reports of fossil Justicieae significantly predate this young fossil (i.e., late Quaternary); not ranked "1" because the image provided does bear resemblance to Justicieae.
OW <i>Justicia/Monechma</i> (Justicieae)	34	1	Fossil not used in analyses.	Ranked "1" because other reports of fossils Justicieae significantly predate this very young fossil (i.e., Holocene); moreover, no image was provided.
pseudocolpate Ruellieae	35	3	Fossil not used in analyses.	Ranked "3" because image provided appears to be correctly identified; fossil arbitrarily down-ranked with respect to fossil #36 because it is of the same age and dates the same clade (fossil #35 is also from the Ziguinchor core).
pseudocolpate Ruellieae Fossil Taxon: not named, but compared to several modern Ruellieae genera	36	4	Fossil attributable to clade of Ruellieae that shares pollen with pseudocolpi (Tripp et al. 2013); used to constrain the MRCA of the six taxa herein sampled that are in that clade.	Ranked "4" because image provided appears to be correctly identified; not ranked as "5" because of concern for possible contamination of its source, (the Ziguinchor core); this fossil was arbitrarily chosen over fossil #35.
<i>Petalidium</i> (Ruellieae)	37	1	Fossil not used in analyses.	Ranked "1" because other fossil Ruellieae predate this very young fossil (i.e., Holocene); moreover, no image was provided. (Gil-Romera et al. 2006) report an increase in Acanthaceae pollen and a decrease in Poaceae pollen in the last 1000 years correlated with increased aridity in Namibia.
<i>Ruellia</i> (Ruellieae)	38	1	Fossil not used in analyses.	Ranked "1" because other fossil Ruellieae predate this very young fossil (i.e., Holocene); moreover, no image was provided. (Gil-Romera et al. 2006) report an increase in Acanthaceae pollen within the last 1000 years, which corresponds to a decrease in Poaceae pollen and drying atmospheric conditions in Namibia.
<i>Ruellia</i> (Ruellieae)	39	2	Fossil not used in analyses.	Ranked "2" owing to uncertain identification; Kuyl et al. (1955) provided a series of plates of reliably dated but mostly unidentified fossils including this one. (Raj 1961) discovered Kuyl's publication (e.g., see fossil #15 above) and attributed Kuyl's Plate 4, Figure 2 to <i>Ruellia</i> ; we are less certain.
<i>Ruellia</i> (Ruellieae)	40	2	Fossil not used in analyses.	Ranked "2" because we were not convinced of taxonomic assignment and no image was provided.
<i>Ruellia</i> (Ruellieae)	41	2	Fossil not used in analyses.	Ranked "2" because the fossil reported in (Frederiksen et al. 1983) is not ascribable to <i>Ruellia</i> with confidence.
Trichantherinae (Ruellieae)	42	1	Fossil not used in analyses.	Ranked "1" because based on an earlier report and extremely young (i.e., Quaternary); moreover, no image was provided.

(continued)

Taxon	#	U	Synapomorphy justification	Utility score justification
Trichantherinae (Ruellieae) Fossil Taxon: not named, but compared to modern <i>Bravaisia</i>	43	5	Pollen of Trichantherinae (except <i>Louteridium</i> ; (Tripp et al. 2013)) is arguably the most distinctive of any Acanthaceae and among the most distinctive across angiosperms. The synapomorphic bands of pseudocolpi arranged in opposing directions and apertures with sexine lips indicate that this palynomorph can be used to constrain the MRCA of the lineage containing <i>Sanchezia</i> .	Ranked "5" because image provided in (Graham 1976) is an excellent match to modern Trichantherinae (Tripp et al. 2013).
Trichantherinae (Ruellieae)	44	5	Fossil not used in analyses.	Ranked "5" because image provided is an excellent match to modern Trichantherinae (Tripp et al. 2013); not used in analyses because fossil #46 predates it. Fossil #45 merits further study.
Trichantherinae (Ruellieae)	45	5	Fossil not used in analyses.	Kuyl et al. (1955) provided a series of plates of reliably dated but mostly unidentified fossils including this one. (Raj 1961) discovered Kuyl's publication and attributed Kuyl's Plate 5, Figures 7–9 (Figs. 1, 8 and 9 in Raj [1961] in error) to Trichantherinae sensu (Tripp et al. 2013). We agree and thus ranked this fossil as "5". However, we used fossil #43 over this fossil because of the former's much narrower stratigraphic range. Use of fossil #45 should be considered in future analyses.
Trichantherinae (Ruellieae)	46	3	Fossil not used in analyses.	Ranked as "2" because despite discussion of fossils, we have been unable to acquire images of this fossil.
Trichantherinae (Ruellieae)	47	1	Fossil not used in analyses.	Ranked "1" because based on an earlier report) and no image was provided; not used in the present study given its extremely young age (i.e., Quaternary).
Trichantherinae (Ruellieae)	48	2	Fossil not used in analyses.	Ranked "2" because of its extremely old age; fossil is highly reminiscent of pollen of Trichantherinae (see text for extensive discussion).
Trichantherinae (Ruellieae)	49	2	Fossil not used in analyses.	Ranked "2" because of its extremely old age; fossil is highly reminiscent of pollen of Trichantherinae (see text for extensive discussion).
NW <i>Justicia</i> (Justicieae)	50	5	Fossil not used in analyses.	Ranked "5" because image provided appears to be correctly identified; fossil number #32, which constrains the same taxa and is the same age, was arbitrarily chosen over this fossil.
NW pseudocolpate Ruellieae Fossil Taxon: not named, but compared to modern <i>Hygrophila</i>	51	5	Fossil distinctly pseudocolpate with sexine lips surrounding germinal apertures; confidently assigned to the clade of Ruellieae that shares these synapomorphies (Tripp et al. 2013). Because of its geographic source (Haiti), it is likely of a taxon with affinities to modern <i>Dyschoriste</i> (formerly <i>Apassalus</i> ; (Tripp et al. 2013)).	Ranked "5" owing to confident identification based on reproduced image and on (Graham 1990) excellent description; used over others that represent NW pseudocolpate Ruellieae because it is oldest. (Graham 1990) noted affinities of this fossil to that of <i>Hygrophila</i> but pollen of that genus is 4-aperturate whereas this palynomorph is 3-aperturate pointing to affinity with modern <i>Dyschoriste</i> .

APPENDIX 2

Voucher information and Genbank numbers (*psbA-trnH*, *trnL-trnF*, *rps16*, *trnT-trnL*, *trnG-trnS*, *trnG-trnR*, ITS+5.8S – = sequence not obtained) for all accessions used in this study. Taxa are arranged phylogenetically from outgroups through Justicieae (Fig. 3).

Outgroups

Martynia annua L.—trnLF: AF067065, rps16: EU529040, trnTL: EU529107, trnGS: EU528979, trnGR: AF169854, ITS: AF169854, Mexico, *Jenkins 97-149* (ARIZ).
Sesamum indicum L.—psbA: EU531713, trnLF: AF067067, rps16: EU529060, trnTL: EU529125, trnGS:

EU528998, trnGR: JQ7801019, ITS: AF169853, cultivated, Mexico, *Jenkins 97-141* (ARIZ).

Nelsonioideae

Elytraria imbricata (Vahl) Pers.—trnLF: AF061819, rps16: EU529020, trnTL: EU529086, trnGS: EU528957, trnGR: KC118367, ITS: AF169852, Arizona, USA, *McDade & Jenkins 1155* (ARIZ).

Nelsonia canescens (Lam.) Spreng.—psbA: KJ140523, trnLF: AF363668, rps16: EU529047, trnTL: EU529114, trnGS: EU528985, ITS: JQ691822, Panama, *Daniel et al. 5254* (CAS).

Staurogyne letestuana Benoist—psbA: KC420678, trnLF: KC420626, rps16: EU529061, trnTL: EU529126, trnGS: EU528999, trnGR: EU528999, ITS: JX443805, cultivated, Belgium, *NBG-B 200000119-77* (BR).

Avicennia lineae

Avicennia bicolor Standl.—psbA: KC420633, trnLF: AY008818, rps16: EU529007, trnTL: EU529072, trnGS: EU528943, trnGR: JQ780995, ITS: EU528877, Costa Rica, *Borg 10* (S).

Avicennia germinans (L.) Stearn—psbA: KC420634, trnLF: AY008819, rps16: EU529008, trnTL: EU529073, trnGS: EU528944, trnGR: KC118339, ITS: EU528878, cultivated, University of Connecticut greenhouses.

Avicennia marina (Forssk.) Vierh.—trnLF: JQ728990, rps16: EU529009, trnGS: EU528945, trnGR: KC118340, ITS: EU528879, Australia, *Schwartzbach 97-A1* (KE).

Thunbergioideae

Mendoncia cowanii (S. Moore) Benoist—trnLF: EU528935, rps16: EU529042, trnTL: EU529109, trnGS: EU528981, ITS: EU528899, Madagascar, *Hearn Mad-3* (PH).

Mendoncia glabra (Poepp. & Endl.) Nees—psbA: KC420647, trnLF: KC420613, rps16: EU529043, trnGS: EU528982, trnGR: EU528982, Bolivia, *Daniel & Wood 10176* (CAS).

Mendoncia phytocrenoides Benoist—trnLF: AF167330, rps16: EU529044, trnTL: EU529111, trnGS: EU528983, trnGR: JQ7801005, ITS: AF169849, Cameroon, *Schönenberger 50* (K).

Thunbergia alata Bojer ex Sims—trnLF: AF061820, rps16: EU529063, ITS: AF169850, cultivated, from commercial seed (ARIZ).

Thunbergia erecta (Benth.) T. Anderson—trnLF: AF061821, rps16: EU529065, trnTL: EU529132, trnGS: EU529001, trnGR: KC118454, ITS: AF169851, cultivated, Missouri Botanical Garden, *Missouri 802421* (MO).

Acantheae

Acanthus sennii Chiov.—psbA: KC420629, trnLF: DQ059148, rps16: DQ054856, trnTL: DQ059224, trnGS:

EU528941, trnGR: KC118335, ITS: DQ028415, Ethiopia, *Friis et al. 7006* (C).

Acanthopsis disperma Nees—psbA: KC420628, trnLF: KC420610, rps16: DQ059142, trnTL: KC118457, trnGS: DQ059218, trnGR: KJ140525, South Africa, *Balkwill et al. 11780* (J).

Aphelandra boyacensis Leonard—psbA: KC420631, trnLF: AF061828, rps16: DQ059196, trnTL: EU529070, trnGS: DQ059275, trnGR: KC118337, ITS: AF169759, Colombia, *McDade 989* (DUKE).

Aphelandra leonardii McDade—psbA: KC420632, trnLF: AF063112, rps16: DQ059205, trnTL: EU529071, trnGS: DQ059287, trnGR: JQ780994, ITS: AF169761, Panama, *McDade 310* (DUKE).

Blepharis maderaspatensis (L.) B. Heyne ex Roth—trnLF: DQ059160, rps16: DQ059160, trnTL: EU529077, trnGS: DQ059237, trnGR: KC118348, ITS: DQ028423, South Africa, *McDade et al. 1292* (PH).

Blepharis subvolubilis C.B. Clarke—trnLF: DQ054864, rps16: DQ059164, trnGS: DQ059241, trnGR: KC118349, ITS: DQ028417, South Africa, *Balkwill et al. 10850* (J);

Crossandra greenstockii S. Moore—psbA: KC420638, trnLF: DQ054871, rps16: DQ059171, trnTL: DQ054871, trnGS: DQ059250, trnGR: KC118352, ITS: DQ028427, South Africa, *McDade & Balkwill 1241* (J);

Geissomeria longiflora Lindl.—psbA: KC420639, trnLF: DQ054904, rps16: DQ059211, trnTL: KC118475, trnGS: DQ059293, trnGR: KC118369, Brazil, *Wasshausen 2337* (US).

Schlerochiton harveyanus Nees—trnLF: DQ054866, rps16: DQ059167, trnTL: KC118506, trnGS: DQ059244, trnGR: KC118446, South Africa, *Balkwill 12274* (J).

Stenandriopsis guineensis (Nees) Benoist—psbA: KC420679, trnLF: DQ054877, rps16: DQ059179, trnTL: EU529127, trnGS: DQ059258, trnGR: JX443969, ITS: DQ028434, cultivated, Royal Botanic Gardens Kew, *K1990-2299* (K).

Stenandrium pilosulum (S.F. Blake) T.F. Daniel —trnLF: AF061827, rps16: DQ059191, trnTL: EU529128, trnGS: DQ059270, trnGR: KC118447 (*Van Devender & Reina 97-454* ARIZ), ITS: AF169758, Mexico, *Van Devender & Reina 97-434* (ARIZ).

Barlerieae

Acanthostelma thymifolium (Chiov.) Bidgood & Brummitt—trnLF: EU528912, rps16: EU529004, trnTL: EU529068, trnGS: EU528939, trnGR: KC118334, ITS: EU528874, Somalia, *Thulin et al. 10650* (UPS);

Acanthura mattogrossensis Lindau—trnLF: EU528913, rps16: EU529005, trnTL: EU529069, trnGS: EU528940, ITS: EU528875, Bolivia, *Bruderreck 173* (K).

Barleria lupulina Lindl.—trnLF: AF289758, rps16: EU529010, trnTL: EU529075, trnGS: EU528946, trnGR: JQ780996, ITS: AF169751, cultivated, San Francisco Conservatory of Flowers, *Daniel s.n.* (CAS).

Barleria repens Nees—trnLF: EU528915, rps16: EU529011, trnTL: EU529076, trnGS: EU528947, trnGR: KC118341, ITS: AF169750, cultivated, Missouri Botanical Garden, *Missouri 97003* (MO).

Crabbea acaulis N.E. Br.—trnLF: EU528918, rps16: EU529016, trnTL: EU529082, trnGS: EU528953, trnGR: JQ781000, ITS: EU528885, South Africa, *Balkwill et al.* 11649 (J).

Golaea migiurtina Chiov.—trnLF: EU528921, rps16: EU529022, trnTL: EU529088, trnGS: EU528959, trnGR: KC118370, ITS: EU528888, Somalia, *Thulin et al.* 10665 (UPS).

Lasiocladus villosus Benoist—trnLF: EU528927, rps16: EU529032, trnTL: EU529098, trnGS: EU528969, trnGR: KC118383, ITS: EU528892, Madagascar, *Daniel et al.* 10427 (CAS).

Lepidagathis alopecuroidea (Vahl) R. Br. Ex Griseb.—trnLF: AF167702, trnGS: EU528971, trnGR: KC118384, ITS: AF169753, Panama, *Daniel et al.* 8066 (CAS).

Lepidagathis formosensis C.B. Clarke ex Hayata—trnLF: EU528930, rps16: EU529034, trnTL: EU529101, trnGS: EU528973, trnGR: KC118385, ITS: EU528895, Taiwan, *Bartholomew et al.* 7654 (CAS).

Lepidagathis scabra C.B. Clarke—trnLF: EU528931, rps16: EU529035, trnTL: EU529102, trnGS: EU528974, trnGR: KC118386, ITS: EU528896, South Africa, *McDade & Balkwill* 1238 (J).

Lophostachys chiapensis Acosta Cast.—trnLF: EU528932, rps16: EU529036, trnTL: EU529103, trnGS: EU528975, trnGR: KC118387, ITS: EU528897, Mexico, *Daniel* 8374 (CAS) multifaceted.

Lophostachys uxpanapensis Acosta Cast.—trnLF: EU528934, rps16: EU529038, trnTL: EU529105, trnGS: EU528977, trnGR: KC118388, ITS: EU528898, Mexico, *Garcia* 539 (ARIZ).

Andrographideae

Andrographis paniculata (Burm. f.) Nees—trnLF: EU528914, rps16: EU529006, trnGS: EU528942, trnGR: JQ7801001, cultivated, *McDade* 1329 (RSA-POM).

Cystacanthus turgidus G. Nicholson—trnLF: EU528919, rps16: EU529017, trnTL: EU529083, trnGS: EU528954, trnGR: KJ140526, ITS: EU528886, cultivated, Royal Botanic Gardens Kew, 1996–479 (K).

Gymnostachyum ceylanicum Arn. & Nees—trnLF: EU528922, rps16: EU529023, trnTL: EU529089, trnGS: EU528960, cultivated, San Francisco Conservatory of Flowers, *Daniel s.n.* (CAS).

Indoneesiella echioides (L.) Sreem. —trnLF: EU528923, rps16: EU529026, trnTL: EU529092, trnGS: EU528963, trnGR: KC118378, cultivated, San Francisco Conservatory of Flowers, *Daniel s.n.* (CAS).

Phlogacanthus thyrsiflorus Nees—trnLF: EU528938, rps16: EU529053, trnTL: EU529120, trnGS: EU528993, trnGR: KC118401, ITS: EU528907, India, *Lindburg* 200 (DAV).

Whitfieldieae

Camarotea souiensis Scott-Elliot—psbA: KC420635, rps16: EU529013, trnTL: EU529079, trnGS: EU528949,

trnGR: JQ780998, ITS: EU528881, Madagascar, *Decary s.n.* (US).

Chlamydocanthus dichrostachyus Mildbr. —psbA: KC420636, trnLF: KC420611, rps16: KC420527, trnTL: EU529080, trnGS: EU528950, trnGR: KC118351, ITS: EU528882, Tanzania, *Semsei* 804 (BR).

Chlamydocanthus euphorbioides Lindau—psbA: KJ140521, trnLF: EU528917, rps16: EU529014, trnTL: EU529081, trnGS: EU528951, trnGR: JQ780999, Madagascar, *Daniel et al.* 10445 (CAS).

Forcipella sp. Baill. —psbA: TCCACAAATGGATA AGACTTGGTCTTAGTGTATAGGATTGAAAATCAAA NCACACAAGGAGCATNAATTCTCTTGATAGAACAA GAAAGAGTTTTATTGCTCCTTAATTTCTTTTCAATTA GTATTGNTTTTTAGTNNGNTGTACTTAGCTNGACTT TTCTTCTTTC (not eligible for GenBank accession number because <200 bp, trnLF: EU528920, rps16: EU529021, trnTL: EU529087, trnGS: EU528958, trnGR: KC118368, ITS: EU528887, Madagascar, *Daniel et al.* 10432 (CAS).

Lankesteria brevior C.B. Clarke—psbA: KC420642, trnLF: AF194436, rps16: KC420529, trnGS: EU528966, trnGR: KC118380, Ghana, *Manktelow et al.* 117 (UPS).

Lankesteria elegans T. Anderson—psbA: KC420643, EU528925, EU529030, EU529096, EU528967, in process, EU528890, Cameroon, *Etuge & Thomas* 466 (CAS).

Lankesteria glandulosa Benoist—psbA: KC420644, EU528926, EU529031, EU529097, EU528968, in process, EU528891, Madagascar, *Daniel et al.* 10435 (CAS).

Leandriella oblonga Benoist—psbA: KC420645, trnLF: EU528928, trnTL: EU529099, trnGS: EU528970, ITS: EU528893, Madagascar, *DuPuy et al.* MB767 (P).

Whitfieldia elongata (P. Beauv.) De Wild. & T. Durand—trnLF: AF195518, rps16: EU529066, trnTL: KC118514, trnGS: EU529002, trnGR: KC118455, ITS: EU528910, cultivated, Uppsala Botanical Garden, *Manktelow* 682 (UPS).

Neuracanthus lineage

Neuracanthus africanus T. Anderson ex S. Moore—psbA: KC420648, trnLF: EU528936, rps16: EU529048, trnTL: EU529115, trnGS: EU528986, trnGR: KC118390, ITS: EU528900, South Africa, *McDade et al.* 1258 (J).

Neuracanthus ovalifolius (Fiori) Bidgood & Brummitt—psbA: KC420649, trnLF: EU528937, rps16: EU529049, trnTL: EU529116, trnGS: EU528988, trnGR: JX443951, ITS: EU528902, Somalia, *Friis et al.* 5032 (K).

Neuracanthus umbraticus Bidgood & Brummitt—psbA: KJ140524, trnLF: KC420615, rps16: EU529051, trnTL: EU529118, trnGS: EU528991, trnGR: JQ7801006, ITS: EU528905, cultivated, San Francisco Conservatory of Flowers, *Daniel* 6770.5 cv (CAS).

Ruellieae

Acanthopale confertiflora (Lindau) C.B. Clarke—JQ7801035, rps16: KC420526, trnTL: KC118456, trnGS:

JQ7801022, trnGR: EF214651, ITS: EF214411, Madagascar, Phillipson 2117 (MO).

Dyschoriste albiflora Lindau—psbA: GA995666, trnLF: KC420612, rps16: KC420528, trnTL: KC118361, trnGS: GQ995605, trnGR: EF214606, ITS: EF214416, Zambia, Luwiika et al. 580 (MO).

Hygrophila corymbosa Lindau—psbA: KC420640, trnLF: AF063120, rps16: EU529024, trnTL: EU529090, trnGS: EU528961, trnGR: KC118371, ITS: AF169836, cultivated, Missouri Botanical Garden, MO897223 (MO).

Mimulopsis solmsii Schweinf. —trnLF: KC420614, rps16: KJ140520, trnTL: KC118485, trnGS: KF945573, trnGR: EF214616, ITS: EF214427, Uganda, ATBP 530 (MO).

Pararuellia alata H.P. Tsui—JX443875, trnLF: KC420616, rps16: KC420530, trnTL: KC118486 (*Zhiduan* 960432 (MO)), trnGR: EF214623, ITS: EF214434, China, *Zhiduan* 960476 (MO).

Phaulopsis imbricata Sweet. —psbA: KC420651, trnLF: KC420617, rps16: KC420531, trnTL: KC118395, trnGS: JX444034, trnGR: EF214619, ITS: EF214430, Tanzania, Bidgood et al. 4589 (MO).

Ruellia humilis Nutt—psbA: GQ995632, trnLF: AF482604 (*S.R. Hill* 30665 (NY)), rps16: AF482538 (*S.R. Hill* 30665 (NY)), trnTL: KC118505, trnGS: EU431038, trnGR: EF214678, ITS: EF214508, Pennsylvania, Tripp 14 (PH).

Sanchezia speciosa Leonard—trnLF: AF063113, rps16: EU529059, trnTL: EU529124, trnGS: EU528997, trnGR: EU431005, ITS: AF169835, cultivated, Duke University Greenhouses, McDade 1180 (ARIZ).

Strobilanthes dyeriana Mast. —psbA: JX443892, trnLF: AF195519, rps16: EU529062, trnTL: EU529129, trnGR: JX443973, ITS: EU528908, cultivated, McDade 1328 (RSA-POM).

Justiceae (psbA and GR are new for this study)

Anisacanthus thurberi (Torr.) A. Gray—psbA: KC420630, Arizona, USA, *Jenkins* 05-007 (ARIZ), rps16: EU087483, trnTL: EU081113, trnGS: EU081046, trnGR: KC118336, Arizona, USA, *Jenkins* 05-007 (ARIZ); trnLF: AF063122, ITS: AF16984, *Van Devender* 88-150 (ARIZ).

Dicliptera extenta S. Moore—trnLF: AF289724, rps16: EU529018, trnTL: EU529084, trnGS: EU528955, ITS: KC420541, cultivated, private garden, South Africa, McDade 1306 (J).

Ecbolium syringifolium (Vahl) Vollesen—trnLF: AF289743, rps16: EU087529, trnTL: DQ372435, trnGS: DQ372480, trnGR: KJ140527, ITS: AF289786, Madagascar, *Daniel & Butterwick* 6733 (CAS).

Justicia adhatoda L. —psbA: KC420641, trnLF: AF289734, rps16: DQ059214, trnTL: EU081176, trnGS: EU081109, trnGR: KC118379, ITS: AF289773, cultivated, University of Arizona Campus Arboretum, *Barr* 60-393 (ARIZ).

Justicia caudata A. Gray—psbA: KJ140522, trnLF: AF063134, rps16: EU529028, trnTL: EU529093, trnGS:

EU528964, trnGR: KJ140528, ITS: AF169837, Faivre 64 (ARIZ).

Mackaya bella Harv. —psbA: KC420646, trnLF: AF289751, rps16: EU529039, trnTL: EU529106, trnGS: EU528979, trnGR: JQ7801003, ITS: AF289796, cultivated, Strybing Arboretum, San Francisco, USA, *Daniel s.n.* (CAS).

Metarungia galpinii (Baden) Baden—trnLF: AF289737, rps16: EU529046, trnTL: EU529113, trnGS: EU528984, ITS: AF289776, South Africa, *Daniel* 9323 (CAS).

Odontonema tubaeforme Kuntze—trnLF: AF063127, rps16: DQ059215, trnTL: DQ372462, trnGS: DQ059297, trnGR: KC118393, ITS: AF169748, cultivated, Duke University Greenhouses, McDade 1182 (ARIZ).

Ptyssiglottis pubisepala (Lindau) B. Hansen—trnLF: AF289744, rps16: EU529055, trnTL: DQ372438, trnGS: DQ372483, trnGR: KC118416, ITS: AF289787, Papua New Guinea, *Daniel* 6630 (CAS).

Rhinacanthus gracilis Klotzsch—psbA: KC420677, trnLF: AF289727, rps16: EU529057, trnTL: EU529122, trnGS: EU528995, trnGR: JQ7801009, ITS: AF289766, cultivated, San Francisco Conservatory of Flowers, *Daniel s.n.* (CAS).

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