Systematic Botany (2002), 27(1): pp. 84–98

Phylogenetic Relationships of the Mangrove Family Avicenniaceae Based on Chloroplast and Nuclear Ribosomal DNA Sequences

ANDREA E. SCHWARZBACH[1] and LUCINDA A. MCDADE[2,3]

[1]Department of Biological Sciences, Kent State University, Kent, Ohio 44242;
Author for correspondence (aschwarz@kent.edu);
[2]Departments of Ecology, Evolutionary Biology, and Plant Sciences, University of Arizona, Tucson, Arizona 85721;
[3]Present address: Department of Botany, Academy of Natural Sciences, Philadelphia, Pennsylvania 19103

Communicating Editor: Aaron Liston

ABSTRACT. Chloroplast (*rbcL, trnL* intron, *trnL-trnF* spacer) and nuclear ribosomal (ITS1, 5.8S, ITS2) DNA sequences were analyzed to identify the closest terrestrial relatives of the mangrove family Avicenniaceae. These plants have been classified within or near Verbenaceae in most synoptic treatments of angiosperms. Surprisingly, Avicenniaceae were placed as part of Acanthaceae s.l. in analyses of all data sets, using both parsimony and maximum likelihood. Within Acanthaceae s.l., our analyses consistently placed *Avicennia* as sister to Thunbergioideae but without strong support. Constrained maximum likelihood analyses indicated that alternative placements of *Avicennia* near the base of Acanthaceae s.l. were not significantly less likely than the sister group relationship with Thunbergioideae. However, placement with Verbenaceae was significantly less likely, as was placement with Pedaliaceae. Morphological evidence is reviewed in this phylogenetic context, and we suggest that articulated nodes and inflorescence structure (including flowers subtended by a bract and two bracteoles) may provide synapomorphies for *Avicennia* and Acanthaceae s.l. We can identify no clear morphological synapomorphies linking *Avicennia* to Verbenaceae. *Avicennia* shares a number of features with each of its putative relatives that are likely to be symplesiomorphic or are of uncertain phylogenetic status.

The Black Mangrove family, Avicenniaceae Endl., includes a single genus, *Avicennia* L., with eight species. Plants of *Avicennia* are trees and woody shrubs distributed in coastal and estuarine habitats in tropical and subtropical areas worldwide (Duke 1991). The ability to survive in mangrove habitats, characterized by high salt concentrations, low aeration of waterlogged soil, and frequently changing water levels due to tidal cycles, has clearly evolved several times independently within angiosperms (Ricklefs and Latham 1993). Tomlinson (1986) grouped plants that occur in mangrove habitats into three categories, major, minor, and associates, based upon the degree to which they are restricted to these habitats and their importance in these communities. *Avicennia* is considered a major or ''true mangrove'' element; these plants are endemic to mangrove habitats, play a predominant role in community structure and have the ability to form pure stands (Tomlinson 1986). Additionally, *Avicennia* is the most species-rich and most frost tolerant of all mangrove genera; it is one of only two ''true mangrove'' genera that are distributed along coastal habitats in both the New and Old World.

The genus exhibits several peculiar morphological, physiological, and anatomical characters, some of which are characteristic of ''true mangroves,'' having evolved in parallel in different mangrove lineages. Examples of such mangrove characters include seawater dispersed fruits that are often viviparous, salt tolerance owing to structural and physiological adaptations (e.g., salt excretion glands and selective ion absorption through roots, respectively), and specialized pneumatophore roots. In addition to these convergent characters, *Avicennia* has unique secondary growth, producing regular growth rings by successive cambia (Zamski 1979; Carlquist 1992).

The large number of convergent and autapomorphic characters has made it difficult to classify *Avicennia* within angiosperms. Van Tieghem (1898) suggested a relationship with Santalaceae based on unspecified embryological similarities, whereas Moldenke (1960) favored Dipterocarpaceae apparently because of similarities between the groups reported to him in a letter from Léon Croizat. Dahlgren (1975) pointed to shared cellular endosperm development in linking Avicenniaceae with Celastraceae. However, most authors have placed *Avicennia* with Asteridae (sensu APG 1998), recognizing that black mangroves share a suite of floral characters with asterids, including sympetalous corollas with epipetalous stamens.

Within asterids, *Avicennia* has been treated either within Verbenaceae (Briquet 1895; Erdtman 1966; Thorne 1976; Cronquist 1981; Reddy et al. 1993) or as a separate family closely related to Verbenaceae (Erdtman 1945; Cantino 1992; Thorne 1992; Takhtajan 1997; Judd et al. 1999), although synapomorphies linking these two groups have never been identified. Recently, *Avicennia* has been included in large-scale molecular analyses using DNA sequences of the chloroplast gene *rbcL* (Wagstaff and Olmstead 1997; Oxelman et al. 1999). These molecular studies clearly establish Avicenniaceae as a member of Lamiales (sensu APG 1998) but, in contrast to previous classifications, suggest that Pedaliaceae (represented by *Sesamum* L.) or Acantha-

ceae are more closely related to Avicenniaceae than Verbenaceae. Although phylogenetic trees produced by these studies are largely unresolved and proposed relationships are not well supported, they provide a foundation for subsequent work.

The goal of the present study was to clarify the phylogenetic relationships of Avicenniaceae, specifically addressing two issues. First, what is the sister group to the mangrove genus *Avicennia* (Avicenniaceae)? Second, can morphological characters be found to support relationships between *Avicennia* and its closest terrestrial relatives, or do the highly specialized characters associated with the mangrove habitat mask these relationships? To address these questions, we examined relationships among species of Avicenniaceae and a number of groups within Lamiales. We included a representative sample of *Avicennia* species based on a biogeographic study that included several populations representing all species and subspecies of *Avicennia* (Schwarzbach and Ricklefs, unpubl. data). We used DNA sequence data from two chloroplast regions, one coding (*rbcL*) and one non-coding (the intron and spacer from the *trnL-trnF* region), and one nuclear region (the nuclear ribosomal internal transcribed spacer region, nr-ITS and 5.8S).

Materials and Methods

Data Gathering Strategy. Earlier phylogenetic work (Wagstaff and Olmstead 1997; Oxelman et al. 1999) included only a single representative of *Avicennia*. Because taxon sampling may affect phylogenetic results, we obtained *rbcL* sequences for three additional species of *Avicennia*, including representatives from both the eastern (Indo-West Pacific) and western (Atlantic, Caribbean and East Pacific) portions of the range of the group. We also included an additional sequence of *Thunbergia* Retz. These were added to a matrix of *rbcL* sequence data that included all taxa placed in the same clade as *Avicennia* in the analysis of Oxelman et al. (1999; i.e., in Oxelman et al.'s Fig. 2, the clade including the labeled groups Acanthaceae [with *Sesamum* in Pedaliaceae basal], Scroph II, and Verbenaceae), plus representatives of other Lamiales (Appendix 1). However, analysis of this matrix indicated that *rbcL* alone is not sufficiently variable to resolve relationships among lineages of Lamiales (see below). Thus our strategy was to obtain sequence data for more rapidly evolving genic regions rather than to acquire more *rbcL* sequences for Lamiales.

The intron and spacer of the *trnL-trnF* region of the chloroplast genome (Taberlet et al. 1991) have been shown to evolve more than twice as rapidly as *rbcL* in one lineage of Lamiales (i.e., Acanthaceae s.l., McDade et al. 2000b; Acanthaceae s.l. includes Nelsonioideae and Thunbergioideae in addition to Acanthaceae s.s.), and to have many informative length mutations and remarkably little homoplasy. For these reasons, we focused sequencing effort on this genic region. From previous work (McDade and Moody 1999; McDade et al. 2000a,b), we had access to a large number of *trnL-trnF* sequences for Acanthaceae s.l. that were generated in the McDade lab. From these, we selected representatives of all major lineages of Acanthaceae s.s. [i.e., Acanthoideae; Barlerieae, Justicieae and Ruellieae from Ruellioideae; classification follows Manktelow et al. (2001)], as well as of Nelsonioideae and Thunbergioideae. We focussed new sequencing effort on Avicenniaceae (as for *rbcL*, representatives from both the eastern and western portions of the range of the group were sequenced), and on other Lamiales, including Pedaliaceae (Appendix 1). Whenever possible, we sought to obtain sequences for species (or congeners) for which *rbcL* sequences were available, reflecting our goal of combining sequence data for multiple genic regions. When this was not possible, we used the results of recent phylogenetic work to select taxa belonging to the same suprageneric lineages as those for which *rbcL* sequences were available. Because resolving relationships within these established suprageneric lineages is not the focus of our study, this sampling strategy should not affect our results.

Sequences for the rapidly evolving nuclear ribosomal internal transcribed spacer region (nr-ITS; Baldwin et al. 1995) are alignable among acanths (McDade et al. 2000b) and between acanths and Avicenniaceae, but only conserved portions of this genic region can be aligned with confidence between these plants and more distant relatives. As a result, our strategy again focused on obtaining sequences for Avicenniaceae. As for the *trnL-trnF* sequences, we had access to a large number of nr-ITS sequences for Acanthaceae s.l. and for *Sesamum* (representing Pedialiaceae) from earlier work (McDade et al. 2000b).

Appendix 1 lists taxa included in this project, along with information regarding which sequences were available from previous work or were generated for this project.

Molecular Methods. Fresh leaf material, leaf material dried in silica gel or, rarely, recently collected herbarium specimens were used as sources of DNA. Total genomic DNA was extracted using the modified CTAB method of Doyle and Doyle (1987). Procedures for purifying genomic DNA and amplifying *rbcL* were as reported in Schwarzbach and Ricklefs (2000); those for the *trnL-trnF* and nr-ITS regions are described in detail by McDade and Moody (1999) and McDade et al. (2000b), respectively. Sequences were generated on ABI automated sequencers using the same primers as in amplification. For most samples, both strands were sequenced for verification and to complete the sequence. Electropherograms of all sequences were proofread manually. Overlapping portions were reconciled by reverse-complementing one, aligning the two, and double-checking any inconsistencies against the electropherograms; mismatches were coded as uncertain.

Alignment and Analysis. Sequences for each genic region were aligned separately by eye in SeqApp 1.9a169 (Gilbert 1992). As noted by McDade and Moody (1999) for Acanthaceae, and by others for other groups (e.g., Gielly et al. 1996; Kim et al. 1996), the *trnL-trnF* sequences have a relatively high frequency of parsimony informative indels. Twenty-three indels were added to the *trnL-trnF* data matrix as presence/absence characters. The indels treated in this way were identified conservatively (i.e., with common 5′ and 3′ termini) and were parsimony informative (i.e., shared by two or more taxa). Numerous short gaps were required to align the nr-ITS sequences. These were almost exclusively in highly variable regions such that they were either not parsimony informative given the relatively sparse taxon sampling employed here or could not be identified conservatively. McDade et al. (2000a) showed that these gaps are informative at considerably lower taxonomic levels than considered here. Further, McDade et al. (2000b) conducted experiments to determine the impact of these hypervariable regions and concluded that they resolve relationships among close relatives and do not obscure phylogenetic signal from more slowly evolving regions that permit resolution of relationships among more distant relatives.

Data matrices for the three genic regions were prepared in MacClade version 4.0a10 (Maddison and Maddison 1999) and are available on request from either author (missing data were 4.2%, 1.1%, and 3.4% for *rbcL*, *trnL-trnF* and nr-ITS, respectively). Preliminary analyses of the separate data sets indicated that the results differed only in degree of resolution or in terms of taxon sampling (results not shown, available from either author). As a result, the sequence data were analyzed in six ways, reflecting our research goal of placing *Avicennia* with confidence (Table 2).

Analysis 1: the *rbcL* data set includes more representatives of Lamiales and thus provides the broadest context for assessing relationships of *Avicennia*. *Analysis 2*: to maximize phylogenetic representation while increasing character evidence (i.e., number of variable sites), we combined the *rbcL* and *trnL-trnF* data; we had sequences for these two regions from essentially the same range

TABLE 1. Characteristics of three genic regions used to place *Avicennia* within Lamiales. To facilitate comparison among loci, statistics reported here are for analyses including only the 20 taxa for which sequences for all three loci were available; note that these values do not match those associated with Figs. 1–4, which depict results of analyses that differed in terms of taxon sampling. — = Indels not scored in the nr-ITS sequences (see text for explanation). [1] Includes 25 and 28 bp of the 18S and 26S ribosomal genes, respectively, that flank ITS1 and ITS2, plus the 5.8S gene.

	rbcL	*trnL-trnF*	nr-ITS region
Aligned length	1428	1231	609 (847)[1]
Variable sites (proportion)	258 (0.18)	422 (0.34)	429 (0.51)[1]
Parsimony informative sites (proportion)	146 (0.10)	233 (0.19)	268 (0.32)[1]
Parsimony informative indels	0	20	—
Pairwise distances (range, %)	0.5–8.3%	0.0–20.6%	0.2–24.4%
Pairwise distances among Acanthaceae s.l. (range, %)	0.5–5.0%	0.0–16.3%	0.2–24.2%
Consistency index	0.650	0.827	0.607
Retention index	0.617	0.757	0.468

of taxa. *Analysis 3*: to maximize character evidence for Acanthaceae s.l. and *Avicennia*, we combined the *trnL-trnF* and nr-ITS data sets. *Analysis 4*: to maximize character evidence for a wide range of Lamiales, including Acanthaceae and Pedaliaceae, we combined all three data sets including taxa for which at least two of three sequences were available. Of 42 taxa in analysis 4, 22 were missing sequence data for one genic region (ca. 17% missing data). Twenty-one of these reflect decisions about data gathering based on relative variability of genic regions: twelve Acanthaceae s.l. lack data for *rbcL* (low intrafamilial variation) and nine Lamiales beyond Acanthaceae and *Avicennia* lack nr-ITS (the region is too variable to be aligned with confidence among distant relatives). No *trnL-trnF* sequence was available for *Clerodendrum*. *Analysis 5*: to maximize character evidence and minimize missing data, we combined all three data sets including only those taxa for which all three sequences were available. Finally, even for the *rbcL* analysis, for which sampling within Acanthaceae s.s. is most sparse, our taxon sample includes more representatives of Acanthaceae than of other Lamiales. *Analysis 6* thus investigated the possible effect of uneven taxon sampling on our results: the data set from analysis 4 (see above) was pruned so that the sample of Acanthaceae s.s. was reduced to three taxa (i.e., equal to the richest sample of other families of Lamiales in this data set).

Matrices were analyzed in PAUP* 4.0b2 (Swofford 2000), with the PAUP* default settings for heuristic searches using parsimony except that addition sequence was set to random with 20 replicates. Multiple most parsimonious (MP) trees were combined as strict consensus trees. For purposes of rooting, *Nicotiana* L. (Solanaceae, Solanales) was included as an outgroup in analyses 2–5 (the *rbcL* data placed *Nicotiana* within Lamiales, perhaps due to long branch attraction). The representative of Oleaceae (*Fraxinus* L. or *Olea* L., depending upon genic region, see Appendix 1) was designated as an outgroup in all analyses; multiple studies of relationships among Lamiales have placed Oleaceae as a basal member of the order (Chase et al. 1993; Soltis et al. 1997).

Strength of support for individual branches was estimated using decay indices (DI; Bremer 1988; Donoghue et al. 1992) and bootstrap values (BS; Felsenstein 1985). DIs for each branch were determined by first using MacClade to prepare a set of trees each with a single branch resolved. These trees were then loaded into PAUP* as constraint trees and the program was asked to find the shortest trees inconsistent with the constraint tree using the same search strategy described above. The difference between the length of these trees and the globally shortest trees is the decay index (DI) for the branch in question. BS values reported are from 200 ''full heuristic'' replicates with ten random sequence addition replicates and TBR branch swapping.

We also conducted maximum likelihood analyses in PAUP* of the data used for parsimony analyses 2 (*rbcL* + *trnL-trnF*) and 3 (*trnL-trnF* + nr-ITS) described above. To reduce search time, the latter matrix was pruned to include a smaller sample of Acanthaceae s.s.; all analyses resolve relationships among Acanthaceae s.s. identically. For both of these analyses, empirical base frequencies were used, the transition: transversion ratio was estimated by the program, and variable sites were set to follow a gamma distribution with four rate categories and the shape parameter set to 0.5. The heuristic search protocol with ten random addition sequences and TBR branch swapping was used. For the ML analysis of the *trnL-trnF* + nr-ITS data, this search strategy did not swap to completion after more than a week. As a result, the search was stopped; the tree produced by this analysis was saved and used as the start tree for a completed search with settings otherwise as above.

Alternative phylogenetic hypotheses were evaluated using MacClade to prepare trees reflecting relationships of interest. For parsimony analysis, these were loaded into PAUP* as constraint trees using the same search strategy described above except that PAUP* was asked to find the shortest trees consistent with the topology in question. The difference between the length of these trees and the globally shortest trees provides an indication of the parsimony cost (in terms of additional evolutionary steps) involved in accepting the alternative hypothesis. For likelihood analysis, constraint trees were loaded in PAUP* and the program was asked to find the most likely tree given the constraint (and the data). Likelihood settings were as described above for the unconstrained searches. Results of the unconstrained analysis were compared to those from analyses with the constraint imposed using the ratio of log likelihood scores; this statistic is distributed as the χ^2 statistic, with degrees of freedom two less than the number of taxa (Sanderson 1997).

RESULTS

Molecular Evolution. In terms of parsimony informative variation, the *trnL-trnF* region is nearly twice as variable as *rbcL*, and the nr-ITS region is half again as variable as *trnL-trnF* (Table 1). Pairwise distance data corroborate this pattern of relative rates of evolution. The lowest pairwise distance value was between species of *Avicennia* for all three genic regions; this is not surprising given that, with four of eight species included in the data sets, this genus was sampled far more densely than any other clade. Table 1 also reports consistency and retention indices for parsimony analyses of data from the three genic regions; for comparability, these were pruned to include only the same 20 taxa for which all three genic regions were sequenced. The *trnL-trnF* data are notably less homoplasious than the other regions and also provide relatively more support for internal nodes as evidenced by the high retention index.

TABLE 2. Phylogenetic relationships of species of *Avicennia* included six analyses; see text for full explanation of strategy in combining data and taxon sampling. The four sampled species of *Avicennia* are monophyletic in all analyses. For each analysis, number and length of most parsimonious (MP) trees, consistency index (CI) and retention index (RI) are also reported; note that these values are from analyses that differed in taxon sampling and thus do not match those presented in Table 1.

Analysis:	Number of taxa	# MP trees, length, CI, RI	Relationships of *Avicennia*
1. *rbcL* (Fig. 1)	41	10 trees, 868, 0.515, 0.571	Polytomy with four lineages of Acanthaceae s.l.
2. *rbcL* + *trnL-trnF* (Fig. 2)	29	3 trees, 1495, 0.681, 0.621	Sister to Thunbergioideae
3. *trnL-trnF* + nr-ITS (Fig. 3)	34	2 trees, 2709, 0.579, 0.596	Sister to Thunbergioideae
4. All three loci: taxa with data for ≥2 loci (Fig. 4)	42	2 trees, 3560, 0.572, 0.585	Sister to Thunbergioideae
5. All three loci: taxa with complete data	20	1 tree, 2289, 0.670, 0.566	Sister to Thunbergioideae
6. All three loci: reduced sample of Acanthaceae s.s.	22	3 trees, 1149, 0.718, 0.563	Sister to Thunbergioideae

Phylogenetic relationships. The four sampled species of *Avicennia* were monophyletic with strong support in all analyses; relationships of the genus are presented by analysis in Table 2. *Avicennia* was placed with Acanthaceae s.l. (i.e., including Thunbergioideae and Nelsonioideae) in all analyses, although not always with full resolution. Further, Acanthaceae s.l. plus *Avicennia* were monophyletic in all analyses, although support for this clade was often weak. No analysis placed *Avicennia* with either Pedaliaceae or Verbenaceae.

Analysis 1,* rbcL *(Fig. 1). The *rbcL* data strongly support monophyly of *Avicennia* (BS=100, DI=9); this lineage is part of a weakly supported (BS=51, DI=1) monophyletic group that includes all Acanthaceae s.l. plus *Avicennia*. Relationships within this group are not resolved, but, in addition to *Avicennia*, Thunbergioideae and Acanthoideae are strongly supported as monophyletic (BS=100, DI=18 and 11, respectively). The other three lineages of Acanthaceae s.s. comprise a monophyletic group, Ruellioideae, but with weak support (BS=62, DI=2). Within Ruellioideae, Justicieae are strongly supported as monophyletic, as are Barlerieae (BS=100 and 96, respectively). Beyond the Acanthaceae s.l. and *Avicennia* lineage, this analysis supports monophyly for most suprageneric lineages of Lamiales that have been established in other analyses (e.g., ''Scroph II'', Lamiaceae, Verbenaceae), but provides essentially no resolution among these lineages. *Schlegelia* Miq. and *Tecoma* Juss. (Bignoniaceae) are not sister taxa but there is only weak support for their placement. Similarly, Pedaliaceae (here represented by *Sesamum* and *Harpagophytum* DC. ex Meissn.) are not monophyletic in this analysis, but there is only very weak support for placement of these genera with other groups (note BS values in Fig. 1). Notably, although support for placement of *Avicennia* with Acanthaceae s.l. is weak, there is no support for placement of this group with Verbenaceae or Pedaliaceae.

Analysis 2,* rbcL + trnL-trnF *(Fig. 2). With more than twice as many parsimony informative characters, this analysis provides better resolution and somewhat stronger support for relationships than the *rbcL* analysis (Fig. 1). *Avicennia* is strongly supported as monophyletic (BS=100, DI=17) and is sister to Thunbergioideae (BS=78, DI=2). This lineage is part of a trichotomy that includes Acanthoideae and Ruellioideae, both of which are strongly supported as monophyletic. These three lineages are together monophyletic with weak support (BS=58, DI=1). Nelsonioideae are sister to other Acanthaceae plus *Avicennia* (BS=74, DI=1). Relationships among other Lamiales are either unresolved or weakly supported except that clades established by previous work are monophyletic (e.g., Lamiaceae, Verbenaceae). Notably, Pedaliaceae are monophyletic with strong support, and are sister to Verbenaceae, with weak support. *Myoporum* and *Leucophyllum* are placed together with remarkably strong support (BS=100, DI=36). Again, there is no indication of a relationship of *Avicennia* to either Pedaliaceae or Verbenaceae.

Analysis 3,* trnL-trnF *+ nr-ITS (Fig. 3). This analysis, which maximized taxon sampling among Acanthaceae s.l., gave results congruent with those from analysis 2 (*rbcL* + *trnL-trnF*; Fig. 2) except that relationships are fully resolved. *Avicennia* is very strongly supported as monophyletic (BS=100, DI=27) and is sister to Thunbergioideae (here including *Mendoncia* Vell. ex Vand. in addition to *Thunbergia*) with weak support (BS=55, DI=2). This lineage is sister to Acanthaceae s.s. with strong support (BS=87, DI=5). Acanthaceae s.s. are monophyletic (BS=61, DI=3), Acanthoideae are sister to Ruellioideae and, within this last clade, Barlerieae are sister to Ruellieae plus Justicieae. Acanthaceae s.l. (including *Avicennia*) are monophyletic, with Nelsonioideae weakly supported as sister to other Acanthaceae plus *Avicennia* (BS=60, DI=2). *Sesamum* (Pedaliaceae) is not part of Acanthaceae s.l.

Analyses 4 and 5, All Three Genic Regions. Results of the analysis combining data for all taxa for which

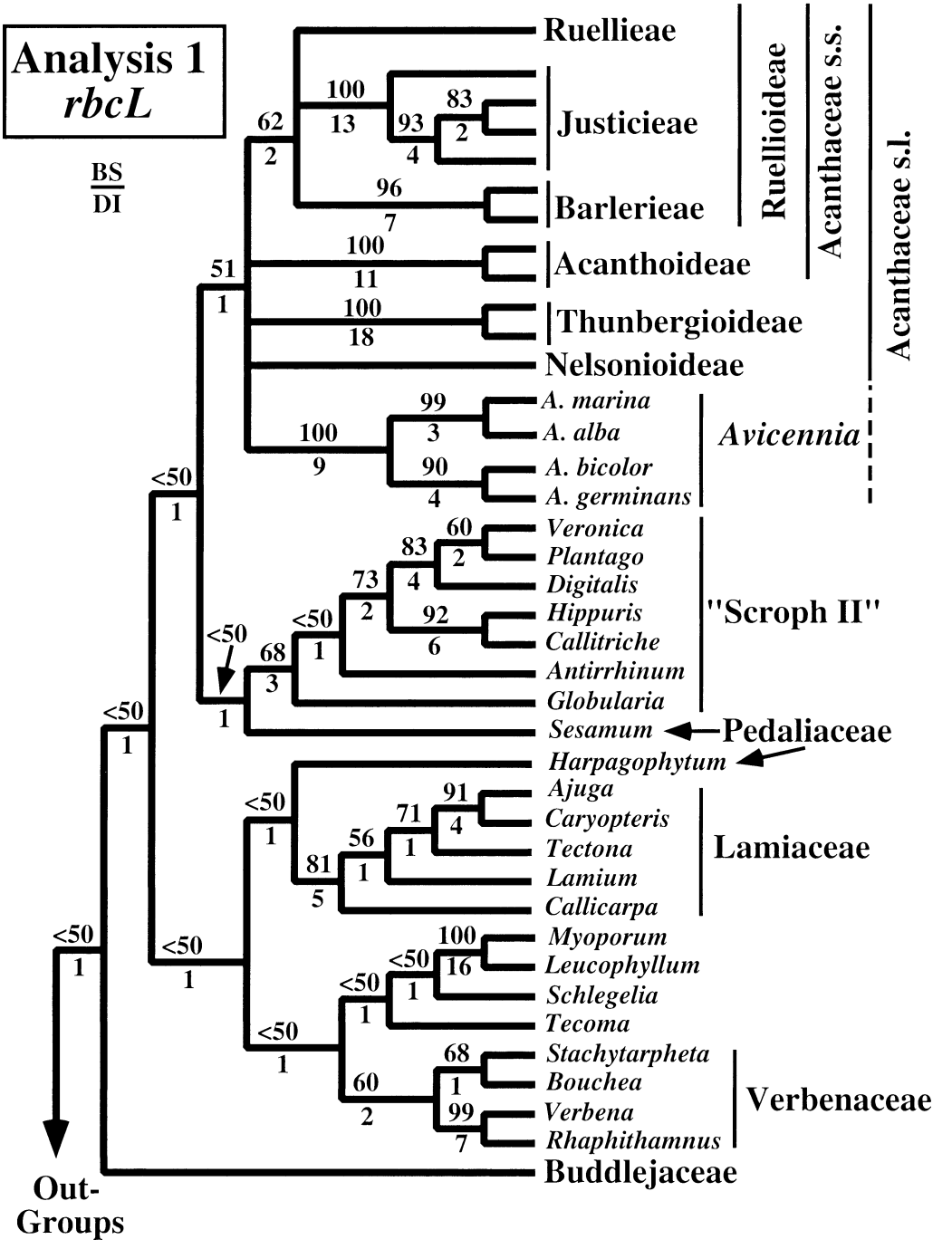

FIG. 1. Strict consensus of ten most parsimonious trees of 868 steps from analysis 1 (*rbcL* alone). CI = 0.515 (excluding uninformative sites), RI = 0.571; of 1428 aligned positions, 1064 are invariant, 208 are parsimony informative. Values above and below the branches are bootstrap and decay indices, respectively. Because sampling within lineages of Acanthaceae s.l. is very sparse, only higher level groups are labeled (see Fig. 4 for results of an analysis including a richer and thus more meaningful sample of Acanthaceae).

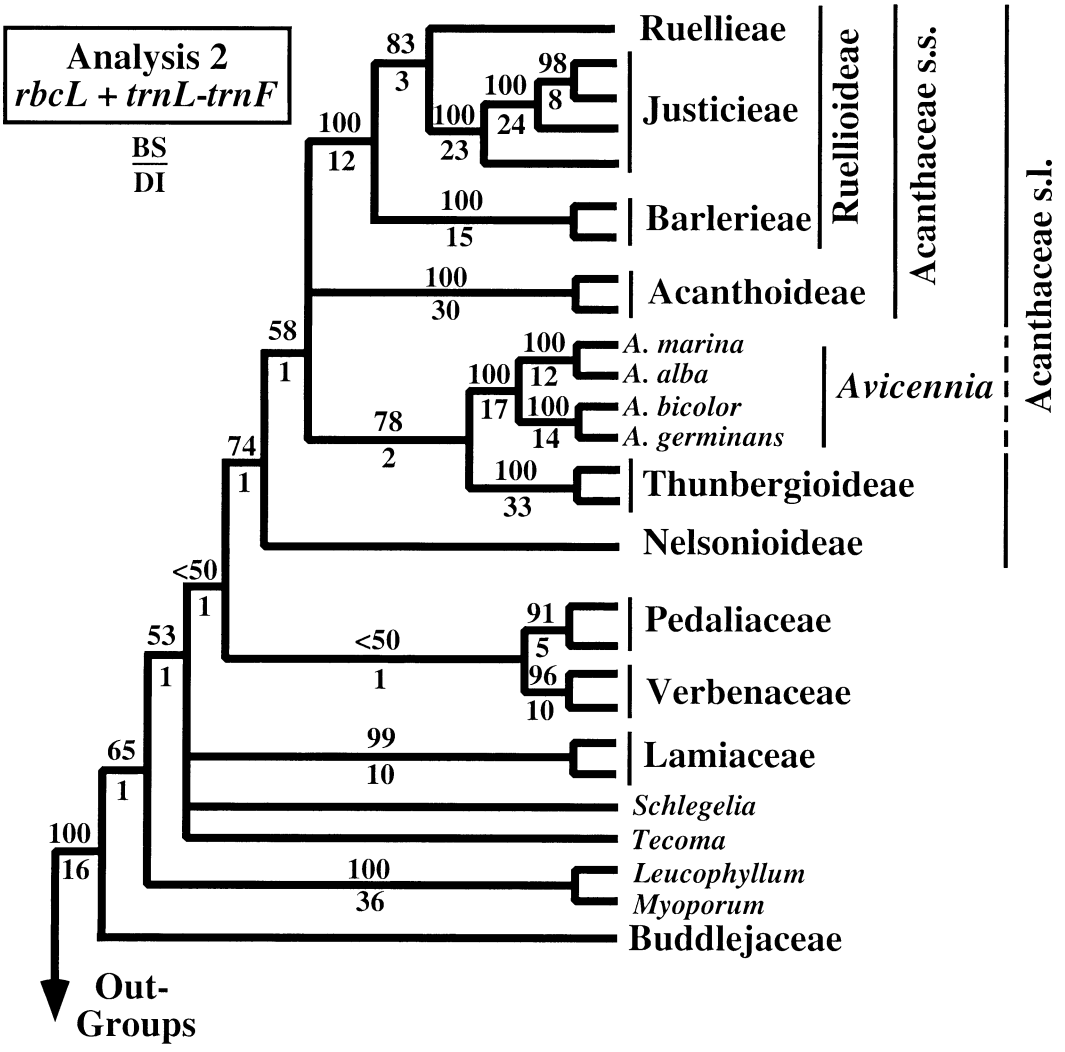

FIG. 2. Strict consensus of three most parsimonious trees of 1495 steps from analysis 2 (*rbcL + trnL-trnF*). CI = 0.681 (excluding uninformative sites), RI = 0.621; of 2633 aligned positions, 1829 are invariant, 404 are parsimony informative. Values above and below the branches are bootstrap and decay indices, respectively. Because sampling within families is very sparse, only higher level groups are labeled with the exception of genera whose placement does not conform to their traditional classification (see Figs. 1 and 4 for results of analyses including richer and thus more meaningful samples of Lamiales and of Acanthaceae, respectively).

sequences were available for at least two of three regions (analysis 4; Fig. 4) are essentially identical to analyses 2 and 3. Again, *Avicennia* is monophyletic with extremely strong support (BS=100, DI=38) and is sister to Thunbergioideae with modest support (BS=62, DI=3). This lineage is sister to Acanthaceae s.s. with strong support (BS=90, DI=7). Acanthaceae s.s. are monophyletic (BS=70, DI=5) and relationships within that lineage are resolved as described above. Nelsonioideae are sister to Acanthaceae s.l. plus *Avicennia* with moderate support (BS=80, DI=3). Relationships among other Lamiales are resolved but with essentially no support except for well-established lineages (i.e., Verbenaceae, Lamiaceae, *Leucophyllum* + *Myoporum*). Pedaliaceae are monophyletic and placed with weak support as sister to Verbenaceae; there is no support for placement of *Avicennia* with these plants.

Restricting the analysis to taxa for which data for all three genic regions were available (analysis 5, results not shown) yielded the same topology as that presented in Figure 4. From this analysis, there is reduced support for monophyly of *Avicennia* plus Thunbergioideae (BS<50, DI=3 compared to BS=62, DI=3 from analysis 4, Fig. 4) and also reduced support for monophyly of all Acanthaceae (including *Avicennia*) except Nelsonioideae (BS=64, DI=3 compared to BS=90, DI=7). However, support for monophyly of Acanthaceae s.l. (includ-

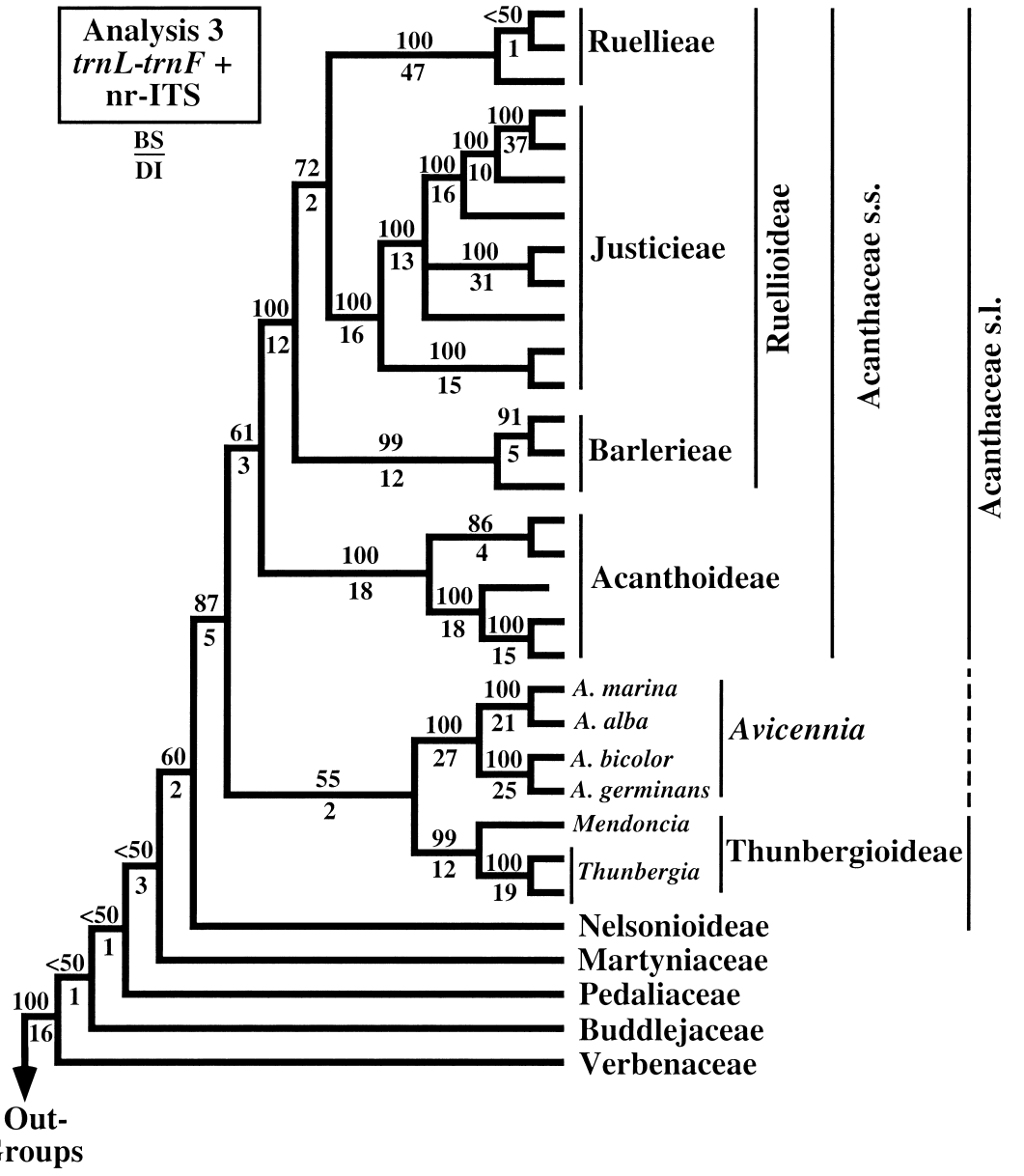

FIG. 3. Strict consensus of two most parsimonious trees of 2709 steps from analysis 3 (*trnL-trnF* + nr-ITS). CI = 0.579 (excluding uninformative sites), RI = 0.596; of 2059 aligned positions, 1050 are invariant, 609 are parsimony informative. Values above and below the branches are bootstrap and decay indices, respectively. To emphasize higher level patterns of relationship, only major lineages of Acanthaceae s.s are labeled (see Fig. 4 for relationships below the tribal level).

ing *Avicennia*) is very strong (BS=93, DI=8), and *Sesamum* (Pedaliaceae) is not part of this group.

Analysis 6, All Three Genic Regions, Acanthaceae s.s. Pruned. Density of taxon sampling within Acanthaceae s.s. had no effect on placement of *Avicennia* (results not shown). As for analyses 2–5, *Avicennia* species were monophyletic and sister to Thunbergioideae but without strong support (BS=56, DI=2). This lineage was part of a polytomy with two lineages of Acanthaceae s.s. Nelsonioideae were not resolved as sister to this entire group in all MP trees, but there was 62% BS support for that relationship.

Maximum Likelihood Analyses. The analysis 2 data set using maximum likelihood gave results only slightly different from parsimony: *Avicennia* is monophyletic and sister to Thunbergioideae but these are together sister to Acanthoideae. However, the branch joining these three lineages is extremely short (0.001 substitutions per site). This analysis also resolves relationships of Lamiales beyond Acanthaceae s.l. and

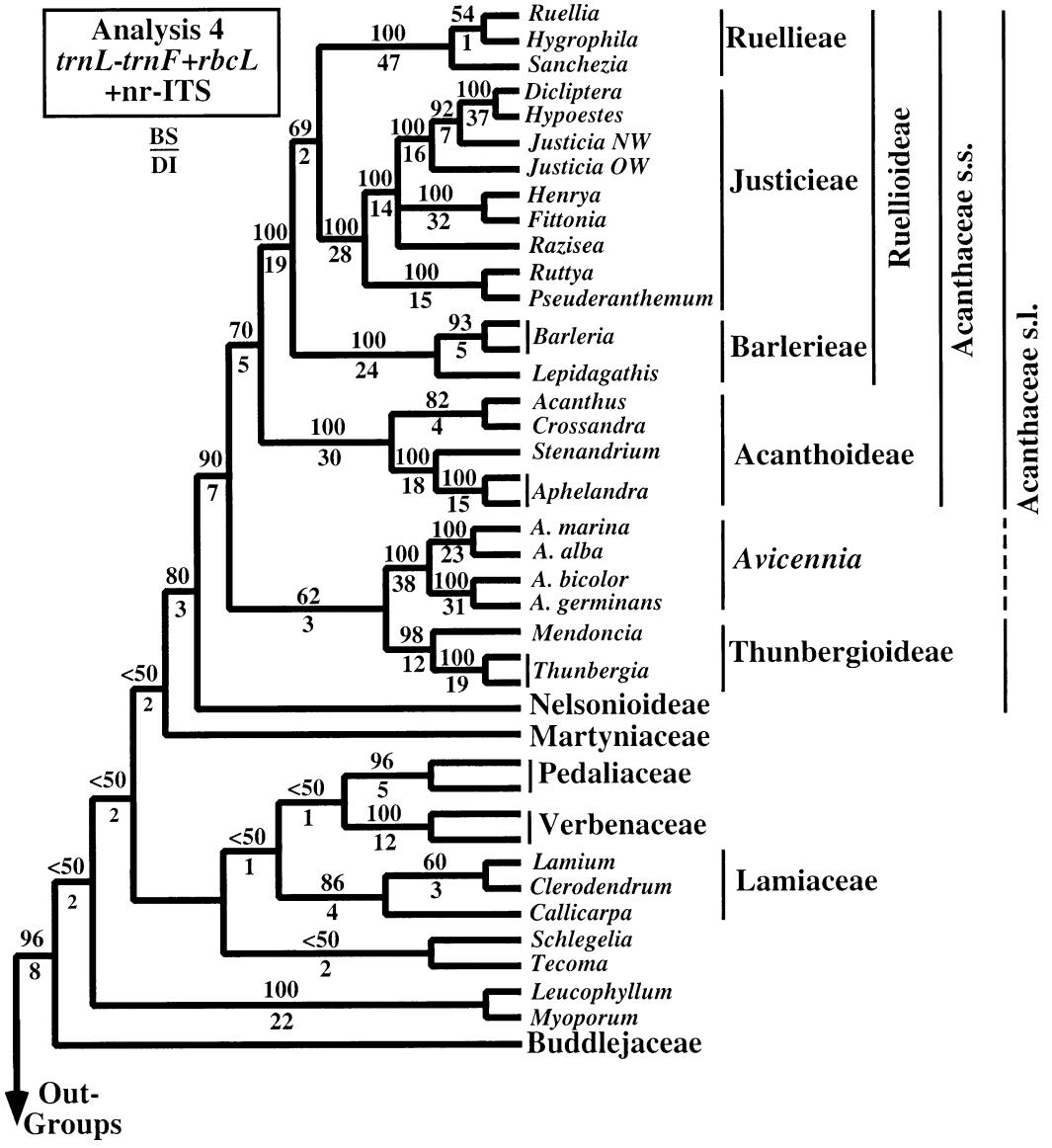

FIG. 4. Strict consensus of two most parsimonious trees of 3560 steps from analysis 4 (all three loci, including taxa for which sequences are available for at least 2 loci). CI = 0.572 (excluding uninformative sites), RI = 0.585; of 3461 aligned positions, 2088 are invariant, 824 are parsimony informative. Values above and below the branches are bootstrap and decay indices, respectively. *Justicia* NW = New World, *Justicia* OW = Old World; see McDade et al. (in press) for details of relationships among Justicieae based on much richer taxon sampling.

Avicennia, but internal branch lengths are extremely short. The analysis 3 data set (*trnL-trnF* + nr-ITS) using likelihood methods yielded a topology identical to parsimony analysis of this data set (Fig. 3) except that the representatives of Buddlejaceae and Verbenaceae switch positions.

Constrained Analyses. Relatively few extra steps were required to achieve the alternative topologies in the constrained parsimony analyses (Table 3). Altering the placement of *Avicennia* within Acanthaceae s.l. (i.e., constraining the genus to be sister to Thunbergioideae, when relevant, or to be sister to Acanthaceae s.s.) required fewer additional steps than removing *Avicennia* from Acanthaceae s.l. or constraining species of *Avicennia* to monophyly with either Pedaliaceae or Verbenaceae.

Constrained maximum likelihood analyses indicated that topologies that forced *Avicennia* to be sister to Acanthaceae s.s. or that forced Acanthaceae s.l. excluding *Avicennia* to monophyly were not less likely than the topologies produced by the unconstrained analyses (Table 4). Interestingly, the latter analysis placed *Avi-*

TABLE 3. Parsimony cost (in terms of number of additional character transitions required) of accepting alternative hypotheses of phylogenetic relationships. Length MP trees = length of trees from unconstrained analysis; remaining columns = additional steps to constrain phylogenetic relationships as indicated (Acanthaceae s.l. monophyletic = Acanthaceae s.s., Thunbergioideae and Nelsonioideae monophyletic exclusive of *Avicennia*). Values of 0 = topology consistent with MP trees; NA = no Verbenaceae available in this data set.

Analysis	Length MP trees	*Avicennia* + *Thunbergioideae*	*Avicennia* + Acanthaceae s.s.	Acanthaceae s.l. monophyletic	*Avicennia* + Pedaliaceae	*Avicennia* + Verbenaceae
1. *rbcL*	868	0	+2 (0.3%)	0	+6 (0.7%)	+4 (0.6%)
2. *rbcL* + *trnL-trnF*	1484	0	+3 (0.2%)	+2 (0.1%)	+11 (0.7%)	+10 (0.7%)
3. *trnL-trnF* + nr-ITS	2709	0	+2 (0.07%)	+10 (0.4%)	+24 (0.9%)	+14 (0.5%)
4. All three loci: taxa with data for ≥2 loci	3560	0	+3 (0.1%)	+10 (0.3%)	+29 (0.8%)	+14 (0.4%)
5. All three loci: taxa with complete data	2289	0	+1 (0.04%)	+1 (0.04%)	+17 (0.7%)	NA

cennia as sister to Acanthaceae s.l. In contrast, constraining the *Avicennia* species to monophyly with representatives of Pedaliaceae or Verbenaceae had significantly lower log likelihood scores than the unconstrained analysis (Table 4). Thus, alternative placements of *Avicennia* in the immediate phylogenetic neighborhood of Acanthaceae s.l. cannot be rejected, but placements with Pedaliaceae or Verbenaceae are significantly less likely.

DISCUSSION

Our results confirm the relative rates of evolution and thus range of phylogenetic utility of the three genic regions with which we worked. Data from these three regions resolve many aspects of relationships of the plants studied here. Nonetheless, it is remarkable that >800 parsimony informative sites from >3400 aligned bases of sequence do resolve relationships among suprageneric groups of Lamiales only with weak support (Fig. 4). This same problem is clear from the work of others using *rbcL* alone (e.g., Wagstaff and Olmstead 1997) and in combination with *ndhF* (Oxelman et al. 1999; note that none of the internal branches in Fig. 4 of Oxelman et al. have jackknife support >50%). It is possible that Lamiales underwent a rapid radiation such that the phylogenetic history of the group is characterized by short internal branches that will be difficult to discover.

On the other hand, molecular data have helped to delimit Lamiales as a whole (Olmstead et al. 1992) and to clarify the phylogenetic status of some lineages within it (e.g., Olmstead and Reeves 1995; Steane et al. 1997; Oxelman et al. 1999; Spangler and Olmstead 1999). In addition to our unexpected results regarding relationships of *Avicennia* (discussed below), our analyses contribute to the on-going process of identifying lineages within Lamiales. Analysis 1 (*rbcL* data alone) confirms the results of earlier analyses of various Lamiales using *rbcL* sequence data regarding delimitation of the ''Scroph II lineage'' of Oxelman et al. (1999) and the placement of a number of genera of traditional Verbenaceae in an expanded Lamiaceae s.l. (Wagstaff and Olmstead 1997). Results from our analyses of combined data sets presented here are either novel or provide independent verification of relationships posited by others. Pedaliaceae are strongly supported as monophyletic in all analyses that included more than one representative of this family except that of *rbcL* data alone. These latter

TABLE 4. Maximum likelihood scores of unconstrained and constrained analyses of two of the combined data sets. Ratio of log likelihood scores is distributed as the χ^2 statistic and tested for significance with degrees of freedom (df) = two less than the number of taxa. For both analyses, number of taxa includes two out-groups; data set 5 was pruned to include only 14 Acanthaceae s.s. (see text for full explanation). df = 27 and 26 for analyses of data sets 4 and 5, respectively.

Analysis of data set	Unconstrained log L	Constrained log L	−2 log likelihood ratio	Reject constraints?
4. *rbcL* + *trnL-trnF*	−12020.05			
Acanthaceae s.s. + *Avicennia*		−12023.32	6.53	No
Acanthaceae s.l. monophyletic		−12025.41	10.78	No
Avicennia + Pedaliaceae		−12055.15	70.19	Yes ($P < 0.001$)
Avicennia + Verbenaceae		−12055.14	70.17	Yes ($P < 0.001$)
5. *trnL-trnF* + nr-ITS	−14256.87			
Acanthaceae s.s. + *Avicennia*		−14259.19	4.65	No
Acanthaceae s.l. monophyletic		−14271.78	29.82	No
Avicennia + Pedaliaceae		−14309.58	105.43	Yes ($P < 0.001$)
Avicennia + Verbenaceae		−14285.97	58.21	Yes ($P < 0.001$)

data, however, provide only weak support for the disparate placements of *Sesamum* and *Harpagophytum* (Fig. 1). The phylogenetic status of Pedaliaceae should be tested with data for additional taxa. Martyniaceae may be the closest relative of Acanthaceae s.l. (i.e., including Nelsonioideae, Thunbergioideae, and *Avicennia*) (Fig. 4), but this relationship is not strongly supported by these data. *Myoporum* (Myoporaceae) and *Leucophyllum* (Scrophulariaceae) are together monophyletic with strong support (Figs. 1, 2, 4). Niezgoda and Tomb (1975) proposed a relationship between these groups based on pollen; in contrast, Carlquist (1992) could not identify features of the wood that would link them. This lineage warrants further study to identify other members and to seek morphological support.

Sequence data from these two chloroplast and one nuclear regions consistently place *Avicennia* with Acanthaceae s.l.; all analyses that resolve relationships further place *Avicennia* as sister to Thunbergioideae. However, the *Avicennia* + Thunbergioideae relationship is not strongly supported in any analysis (maximum support values are BS=78, DI=2). Topologically constrained parsimony analyses indicate that alternate placements of *Avicennia* in the phylogenetic vicinity of Acanthaceae s.l. require few additional steps. Further, maximum likelihood analyses indicate that such placements of *Avicennia* are not less likely than its placement in the unconstrained analyses as sister to Thunbergioideae. However, given these data, placement of *Avicennia* with Pedaliaceae or Verbenaceae is significantly less likely than placement with Acanthaceae s.l.

Evaluating these results in the context of morphological data is problematic because *Avicennia* shares many characteristics with other mangroves presumably as a result of convergent evolution, and also is autapomorphic in some respects. Also problematic is the incomplete status of progress toward identifying monophyletic lineages among Lamiales and resolving relationships among these lineages, as described above. Particularly relevant here is that our present understanding of the delimitation of Verbenaceae and Lamiaceae differs markedly from the traditional delimitation of these groups and this affects interpretation of morphological evidence for relationships of *Avicennia*. Further complicating our assessment of morphological characters is our inability to identify with confidence the next closest relatives of Acanthaceae s.l. We thus lack the phylogenetic context, both in terms of identity of monophyletic lineages and of relationships among them, to undertake an explicit examination of morphological evidence. With these caveats, in the sections that follow, we discuss the morphological basis for relationships of *Avicennia*, with emphasis on comparison of these plants to groups with which they have been associated either in the literature (i.e., Verbenaceae, Pedaliaceae) or in the present study (i.e., Acanthaceae s.l.).

The woody, mangrove habit of *Avicennia* does not associate it clearly with other Lamiales. There have clearly been many evolutionary shifts in habit among these plants and most supra-generic lineages include both herbaceous and woody members. Although woodiness may seem out of place in Acanthaceae, shrubs and small trees are not, in fact, unusual among Acanthaceae s.s. It is also noteworthy that *Trichanthera gigantea* Humb. & Bonpl. ex Steud. (Ruellieae) occurs in riparian habitats and has prop roots, and that two of three species of New World *Bravaisia* DC. (Ruellieae) and at least one species of *Acanthus* L. (i.e., the ''mangrove thistle'', *A. ilicifolius* L., Acanthoideae; this species sometimes split into three different species, *A. ilicifolius*, *A. ebracteatus* Vahl., and *A. volubilis* Wall.) are mangroves (Daniel 1988; Tomlinson 1986). Our results indicate that these plants are not the closest relatives of *Avicennia* within Acanthaceae s.l., but their existence does suggest considerable evolutionary flexibility in habit and habitat within the lineage. Neither habit nor habitat supports placement of *Avicennia* with Thunbergioideae: these latter plants are terrestrial twining vines or scramblers (plants of a few species of *Thunbergia* Retz. are erect). With the possible exception of Acanthoideae, plants of *Avicennia*, Thunbergioideae and Acanthaceae s.s. have articulated stems (i.e., with a notable ring at the nodes; see Tomlinson 1986, fig. B.8.c). To our knowledge, this trait is not common in other Lamiales and does not mark large suprageneric lineages.

In *Avicennia*, secondary growth occurs via successive cambia that form external to the previously active cambium(a) resulting in concentric rings of xylem and phloem (Zamski 1979; Carlquist 1992; see fig. B12 in Tomlinson 1986). Interestingly, Watson and Dallwitz (1992 onwards) indicate that *Afromendoncia* Gilg ex Lindau (Thunbergioideae) also has concentric rings of vascular tissue. However, these apparently form quite differently: *Afromendoncia* has what Carlquist (1988) has called ''centripetal successive cambia'' in which a second series of vascular bundles forms not external to the primary cambium, as in *Avicennia*, but rather internal to it, in the pith. These bundles are ''inverse'' (i.e., they produce phloem centripetally and xylem centrifugally) (Obaton 1960). Carlquist and Zona (1988) did not observe such cambia in the several species of *Mendoncia* Vell. ex Vand. or *Thunbergia* that they studied, nor did Hérail (1885) observe such cambia in two species of *Thunbergia* (one of which was treated as *Hexacentris* Nees, a genus now synonymized with *Thunbergia*). Obaton (1960) also recorded ''inverse bundles in the pith'' in *Mendoncia* and *Pseudocalyx* Radlk. (Thunbergioideae) and in *Acanthus*, but without documentation. Hérail (1885) provided clear documentation of inverse bundles in the pith of three species of *Acanthus*. Interxylary phloem has been reported in species of *Thunbergia* (Hérail 1885; Obaton 1960; Carlquist 1988; Carlquist and Zona 1988). Hérail

(1885) and particularly Obaton (1960) also documented fissuring of the wood in species of Thunbergioideae that are lianas. It is thus apparent that members of Thunbergioideae, as well as *Acanthus*, have a number of forms of anomalous secondary growth. It is less clear whether these provide a basis for linking Thunbergioideae to *Avicennia*, but it is perhaps noteworthy that anomalous wood is not known among Verbenaceae (Carlquist pers. comm.).

Carlquist (1992) found no wood characters linking *Avicennia* to Verbenaceae and, in fact, argued for removal of *Avicennia* from Verbenaceae on this basis. In a survey of wood anatomy of Martyniaceae and Pedaliaceae, Carlquist (1987) links these plants clearly to Scrophulariales (= part of Lamiales sensu Olmstead et al. 1992). He did not explicitly compare woods of Pedaliaceae to those of *Avicennia*, but descriptions provide no basis for linking these two groups.

Like most Acanthaceae s.s., plants of *Avicennia* apparently have inflorescences that are thyrses with the individual cymes often reduced to a single flower. Each flower is subtended by a bract and two bracteoles (see Fig. B.8 in Tomlinson [1986:69]; in describing *Avicennia*, these have been referred to together as a ''pseudo-involucre of bractlets'' [Mabberley 1997]). The phylogenetic status of these characters is unclear for a number of reasons. In Thunbergioideae, each flower is subtended by two bract-like structures that are alike in size and shape. These may be bracteoles, with the bract having been lost, but establishing precise homologies will require developmental work. Plants of Nelsonioideae likely share this inflorescence structure, including the bract and two bracteoles subtending each flower, although *Nelsonia* R.Br. has apparently lost the bracteoles (these have also been lost in a few groups within Acanthaceae s.s.; the basic inflorescence structure has been modified in other ways in this large and diverse group as well). Martyniaceae have similar bracts and bracteoles but they are caducous and flowers are usually pedicellate such that homologies are uncertain (P. K. Bretting pers. comm.). Pedaliaceae apparently have inflorescences quite similar to those of *Avicennia* and Acanthaceae: flowers are bracteate and the lateral flowers of the cymose units are reduced to nectaries (Watson and Dallwitz 1992 onwards). Verbenaceae s.s. have indeterminate inflorescences; flowers are sometimes bracteate but apparently not bracteolate. Establishing the phylogenetic status of these traits clearly requires additional comparative study as well as improved understanding of phylogenetic relationships.

Like most Lamiales, plants of *Avicennia* have bicarpellate ovaries; in addition, there are two ovules per carpel and the ovules are arranged collaterally (i.e., side by side). The four ovules and their collateral arrangement seem to be the key links between *Avicennia* and Verbenaceae. However, a number of other lineages of Lamiales, including Acanthaceae s.s., Thunbergioideae, and Lamiaceae include plants with four ovules (Nelsonioideae have many). The same is true of collateral arrangement of the ovules: plants of Thunbergioideae (Schönenberger and Endress 1998), Lamiaceae, and the more distantly related Boraginaceae share this arrangement. In Nelsonioideae and Acanthaceae s.s., as well as in most other Lamiales (including Pedaliaceae), the ovules are superposed or ''columnar'' in each locule. It seems that both ovule number and arrangement have evolved homoplastically in Lamiales and could link *Avicennia* to either Thunbergioideae or Verbenaceae.

The ovaries of some species of *Avicennia* are partially false septate, but these septa are lacking in other species. To our knowledge, false septa are unknown in Thunbergioideae or Acanthaceae s.s. Ovaries of Verbenaceae (and Lamiaceae) and some Pedaliaceae have partial to complete false septa such that the ovary may become four-locular. In Pedaliaceae, the ovary becomes more strongly septate in fruit. False septa thus occur in diverse lineages of Lamiales and homologies are unclear due to variation in structure and development. In addition, some species of *Avicennia* lack false septa as noted above.

Placentation in *Avicennia* is described by Tomlinson (1986) as ''..essentially axile, the 4 ovules pendulous from a central stalk that has a terminal umbo projecting into the base of the stylar canal without closing it.'' This coincides with descriptions and figures provided by Junell (1934). Acanthaceae s.l. (including Thunbergioideae and Nelsonioideae) have axile placentation, as do Pedaliaceae and Verbenaceae (Martyniaceae have parietal placentation). Placentation type thus links *Avicennia* equally well to all of its putative relatives. The same is true of endosperm: seeds of plants belonging to most of the lineages in question lack endosperm (i.e., *Avicennia*, Acanthaceae s.s., Thunbergioideae, most Verbenaceae and Lamiaceae, some Pedaliaceae). In contrast, seeds of Nelsonioideae have oily endosperm and those of Martyniaceae are ''scantily endospermic'' (Watson and Dallwitz 1992 and onwards).

Ovule orientation would seem to place *Avicennia* with Verbenaceae (both have orthotropous ovules) and to contradict placement with Thunbergioideae which have anatropous ovules, as do Acanthaceae s.s. However, ovule orientation is quite variable among Lamiales and confident assessment of the phylogenetic utility of this character will require improved understanding of relationships.

Interestingly, *Avicennia* (Tomlinson 1986, Fig. B.8.j) and Thunbergioideae have seeds with folded cotyledons (Lindau 1895; Sanders 1997). It is not, however, clear whether the folded cotyledons of Thunbergioideae are homologous to those of *Avicennia*. Seeds of Acanthaceae s.s. with which we are familiar do not share this trait, although Watson and Dallwitz (1992

and onwards) report that some acanths have planoconvex or crumpled cotyledons. Folded cotyledons have not, to our knowledge, been reported from Verbenaceae or Pedaliaceae.

We suggest that morphological links between *Avicennia* and Verbenaceae are ambiguous. Characteristics that these plants share are plesiomorphic, shared with other groups, or are not clearly homologous. Articulated nodes may link Acanthaceae s.s., Thunbergioideae and *Avicennia*; inflorescence structure, including flowers subtended by a bract and two bracteoles, may well link these three groups plus Nelsonioideae although both Martyniaceae and Pedaliaceae require additional study in this regard. The folded cotyledons of *Avicennia* and Thunbergioideae require further study to assess homologies. Other characters of *Avicennia* are at least as readily accommodated in the phylogenetic vicinity of Acanthaceae s.l. as with Verbenaceae. Morphological evidence certainly does not refute placement with Acanthaceae s.l. Based on both molecular and morphological evidence, we thus accept *Avicennia* as part of Acanthaceae s.l. However, more data will be required to place *Avicennia* more precisely; the sister relationship between *Avicennia* and Thunbergioideae is consistently but not strongly supported by our data.

As for other highly autapomorphic mangrove groups (e.g., Schwarzbach and Ricklefs 2000), phylogenetic analysis of molecular data has helped to place Avicenniaceae. Placement with Verbenaceae can be rejected, and a relationship with Acanthaceae s.l. is both consistently and strongly supported by molecular sequence data from three genic regions in two genomes. The consistent but weakly supported result that *Avicennia* and Thunbergioideae are sister groups merits further testing. Perhaps not surprisingly given the combination of convergent and autapomorphic characteristics of *Avicennia*, the morphological data are not as clear. However, if morphological evidence does not unambiguously link *Avicennia* with Acanthaceae or with Thunbergioideae, there is even less basis to link the black mangroves to Verbenaceae. Identifying the closest living relatives of *Avicennia* should facilitate understanding the phylogenetic and ecological contexts in which this intriguing group of plants evolved.

ACKNOWLEDGEMENTS. We thank Robert E. Ricklefs for his generous support, S. Carlquist for insight and advice on wood anatomy, P. Cantino and P. Bretting for helpful discussion of Lamiales, A. E. Arnold, T. F. Daniel, R. A. Levin, and S. Wagstaff for many valuable comments on earlier versions of the manuscript, and K. M. Riley for help in LAM's lab. We are grateful to D. Swofford and D. Maddison for making available test versions of PAUP* and MacClade, respectively. This research was partially supported by grants to LAM from the U.S. National Science Foundation (DEB BSR-8507517, DEB BSR-9707693). The Univ. of Arizona Research Training Group in the Analysis of Biological Diversification (NSF DIR-9113362, BIR-9602246) and University of Arizona's Undergraduate Biological Research Participation program supported undergraduate participation in the molecular work. For help in acquiring plant materials we thank T. F. Daniel, N. C. Duke, W. Hauk, P. Jenkins, J. MacDougal, R. Olmstead, R. E. Ricklefs, J. Schönenberger, D. E. Stone, B. Tankersley, M. Turner, T. Van Devender, P. Wilson, W. Yong; ARIZ, CAS, and MO; and the staff of the Duke Univ. Greenhouses.

LITERATURE CITED

ANGIOSPERM PHYLOGENY GROUP. 1998. An ordinal classification for the families of flowering plants. Annals of the Missouri Botanical Garden 85: 531–553.

BALDWIN, B. G., M. J. SANDERSON, J. M. PORTER, M. F. WOJCIECHOWSKI, C. S. CAMPBELL, and M. J. DONOGHUE. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden 82: 247–277.

BREMER, K. 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstructions. Evolution 42: 795–803.

BRIQUET, J. 1895. Verbenaceae. Pp. 133–182 in *Die natürlichen Pflanzenfamilien*, eds. A. Engler and K. Prantl. Leipzig: W. Engelmann.

CANTINO, P. D. 1992. Evidence for a polyphyletic origin of the Labiatae. Annals of the Missouri Botanical Garden 79: 361–379.

CARLQUIST, S. 1987. Wood anatomy of Martyniaceae and Pedaliaceae. Aliso 11: 473–483.

———. 1988. *Comparative wood anatomy: systematic, ecological, and evolutionary aspects of Dicotyledon wood*. New York: Springer-Verlag.

———. 1992. Wood anatomy of sympetalous dicotyledon families: a summary, with comments on systematic relationships and evolution of the woody habit. Annals of the Missouri Botanical Garden 79: 303–332.

——— and S. ZONA. 1988. Wood anatomy of Acanthaceae: a survey. Aliso 12: 201–227.

CHASE, M. W., D. E. SOLTIS, R. G. OLMSTEAD, D. MORGAN, D. H. LES, B. D. MISHLER, M. R. DUVALL, R. A. PRICE, H. G. HILLS, Y.-L. QIU, K. A. KRON, J. H. RETTIG, E. CONTI, J. D. PALMER, J. R. MANHART, K. J. SYTSMA, H. J. MICHAELS, W. J. KRESS, K. G. KAROL, W. D. CLARK, M. HEDRÉN, B. S. GAUT, R. K. JANSEN, K.-J. KIM, C. F. WIMPEE, J. F. SMITH, G. R. FURNIER, S. H. STRAUSS, Q.-Y. XIANG, G. M. PLUNKETT, P. S. SOLTIS, S. M. SWENSEN, S. E. WILLIAMS, P. A. GADEK, C. J. QUINN, L. E. EGUIARTE, E. GOLENBERG, G. H. LEARN, JR., S. W. GRAHAM, S. C. H. BARRETT, S. DAYANANDAN, and V. A. ALBERT. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbcL*. Annals of the Missouri Botanical Garden 80: 528–580.

CRONQUIST, A. 1981. *An integrated system of classification of flowering plants*. New York: Columbia University Press.

DAHLGREN, R. A. 1975. A system of classification of the angiosperms to be used to demonstrate the distribution of characters. Botaniska Notiser 128: 119–147.

DANIEL, T. F. 1988. A systematic study of *Bravaisia* DC. (Acanthaceae). Proceeding of the California Academy of Sciences 45: 111–132.

DONOGHUE, M. J., R. G. OLMSTEAD, J. F. SMITH, and J. D. PALMER. 1992. Phylogenetic relationships of Dipsacales based on *rbcL* sequences. Annals of the Missouri Botanical Garden 79: 333–345.

DOYLE, J. J. and J. L. DOYLE. 1987. A rapid DNA isolation procedure for small amounts of fresh leaf tissue. Phytochemical Bulletin 19: 11–15.

DUKE, N. C. 1991. A systematic revision of the mangrove genus *Avicennia* (Avicenniaceae) in Australasia. Australian Systematic Botany 4: 299–324.

ERDTMAN, G. 1945. Pollen morphology and plant taxonomy. Svensk Botanisk Tidskrift 39: 279–285.

———. 1966. *Pollen morphology and plant taxonomy.* New York, London: Hafner.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

GIELLY, L., Y.-M. YUAN, P. KÜPFER, and P. TABERLET. 1996. Phylogenetic use of noncoding regions in the genus *Gentiana* L.: chloroplast *trnL* (UAA) intron versus nuclear ribosomal internal transcribed spacer sequences. Molecular Phylogenetics and Evolution 5: 460–466.

GILBERT, D. G. 1992. SeqApp: a biosequence editor and analysis application. Privately published by the author. Indiana University, Bloomington.

HÉRAIL, J. 1885. Recherches sur l'anatomie comparée de la tige des Dicotylédones. Annales des Sciences Naturelles Botanique 7, ser. 2: 203–314.

JUDD, W. S., C. S. CAMPBELL, E. A. KELLOGG, and P. F. STEVENS. 1999. *Plant systematics: a phylogenetic approach.* Sunderland, Massachusetts: Sinauer Associates.

JUNELL, S. 1934. Zur Gynäceumsmorphologie und Systematik der Verbenaceen und Labiaten. Symbolae Botanicae Upsalienses 4: 1–219.

KIM, J. H., H. 'T. HART, and T. H. M. MES. 1996. The phylogenetic position of East Asian *Sedum* species (Crassulaceae) based on chloroplast DNA *trnL* (UAA)-*trnF* (GAA) intergenic spacer sequence variation. Acta Botanica Neerlandica 45: 309–321.

LINDAU, G. 1895. Acanthaceae. Pp. 274–353 in *Die natürlichen Pflanzenfamilien*, eds. A. Engler and K. Prantl. Leipzig, Germany: Engelmann.

MABBERLEY, D. J. 1997. *The plant book.* Cambridge: Cambridge University Press.

MADDISON, W. P., and D. R. MADDISON. 1999. *MacClade.* Sunderland, Massachusetts: Sinauer Associates.

MANKTELOW, M., L. A. MCDADE, B. OXELMAN, C. A. FURNESS, and M.-J. BALKWILL. 2001. The Enigmatic Tribe Whitfieldieae (Acanthaceae): Delimitation and phylogenetic relationships based on molecular and morphological data. Systematic Botany 26: 104–119.

MCDADE, L. A. and M. L. MOODY. 1999. Phylogenetic relationships among Acanthaceae: evidence from non-coding *trnL-trnF* chloroplast DNA sequences. American Journal of Botany 86: 70–80.

———, T. F. DANIEL, S. E. MASTA, and K. M. RILEY. 2000a. Phylogenetic relationships within the tribe Justicieae (Acanthaceae): evidence from molecular sequences, morphology, and cytology. Annals of the Missouri Botanical Garden 87: 435–458.

———, S. E. MASTA, M. L. MOODY, and E. WATERS. 2000b. Phylogenetic relationships among Acanthaceae: evidence from two genomes. Systematic Botany 25: 105–120.

MOLDENKE, H. N. 1960. Materials towards a monograph of the genus *Avicennia.* Phytologia 7: 123–168, 179–232, 259–293.

NIEZGODA, C. J. and A. S. TOMB. 1975. Systematic palynology of the tribe Leucophylleae (Scrophulariaceae) and selected Myoporaceae. Pollen and Spores 17: 495–516.

OBATON, M. 1960. Les lianes ligneuses a structure anormale des forets denses d'Afrique occidentale. Annales des Sciences Naturelles, Botanique 12: 1–220.

OLMSTEAD, R. G., H. J. MICHAELS, K. M. SCOTT, and J. D. PALMER. 1992. Monophyly of the Asteridae and identification of their major lineages inferred from DNA sequences of *rbcL*. Annals of the Missouri Botanical Garden 79: 249–265.

——— and P. A. REEVES. 1995. Evidence for the polyphyly of the Scrophulariaceae based on chloroplast *rbcL* and *ndhF* sequences. Annals of the Missouri Botanical Garden 82: 176–193.

OXELMAN, B., M. BACKLUND, and B. BREMER. 1999. Relationships of the Buddlejaceae s.l. investigated using parsimony jackknife and branch support analysis of chloroplast *ndhF* and *rbcL* sequence data. Systematic Botany 24: 164–182.

REDDY, M. S., C. V. KUMARI, and M. RADHAKRISHNAIAH. 1993. Systematic position of *Avicennia*. Feddes Repertorium 104: 237–239.

RICKLEFS, R. E. and R. E. LATHAM. 1993. Global patterns of diversity in mangrove floras. Pp. 215–229 in *Species diversity in ecological communities: historical and geographical perspectives*, eds. R. E. Ricklefs and D. Schluter. Chicago: University of Chicago Press.

SANDERS, R. W. 1997. The Avicenniaceae in the Southeastern United States. Harvard Papers in Botany 10: 81–92.

SANDERSON, M. J. 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Molecular Biology and Evolution 14: 1218–1231.

SOLTIS, D. E., P. S. SOLTIS, D. L. NICKRENT, L. A. JOHNSON, W. J. HAHN, S. B. HOOT, J. A. SWEERE, R. K. KUZOFF, K. A. KRON, M. W. CHASE, S. M. SWENSON, E. A. ZIMMER, S.-M. CHAW, L. J. GILLESPIE, W. J. KRESS, and K. J. SYTSMA. 1997. Angiosperm phylogeny inferred from 18S ribosomal DNA sequences. Annals of the Missouri Botanical Garden 84: 1–49.

SCHÖNENBERGER, J. and ENDRESS, P. K. 1998. Structure and development of the flowers in *Mendoncia*, *Pseudocalyx*, and *Thunbergia* (Acanthaceae) and their systematic implications. International Journal of Plant Sciences 159: 446–465.

SCHWARZBACH, A. E. and R. E. RICKLEFS. 2000. Systematic affinities of Rhizophoraceae and Anisophylleaceae, and intergeneric relationships within Rhizophoraceae, based on chloroplast DNA, nuclear ribosomal DNA, and morphology. American Journal of Botany 87: 547–564.

SPANGLER, R. E. and R. G. OLMSTEAD. 1999. Phylogenetic analysis of Bignoniaceae based on the cpDNA gene sequences *rbcL* and *ndhF.* Annals of the Missouri Botanical Garden 86: 33–46.

STEANE, D. A., R. W. SCOTLAND, D. J. MABBERLEY, S. J. WAGSTAFF, P. A. REEVES, and R. G. OLMSTEAD. 1997. Phylogenetic relationships of *Clerodendrum* s.l. (Lamiaceae) inferred from chloroplast DNA. Systematic Botany 22: 229–243.

SWOFFORD, D. L. 2000. *PAUP*. Phylogenetic Analysis Using Parsimony (*And Other Methods).* Version 4. Sunderland, Massachusetts: Sinauer Associates.

TABERLET, P., L. GIELLY, G. PAUTOU, and J. BOUVET. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17: 1105–1109.

TAKHTAJAN, A. 1997. *Diversity and classification of flowering plants.* New York: Columbia University Press.

THORNE, R. F. 1976. A phylogenetic classification of the Angiospermae. Evolutionary Biology 9: 35–106.

———. 1992. Classification and geography of the flowering plants. The Botanical Review 58: 225–348.

TOMLINSON, P. B. 1986. *The botany of mangroves.* Cambridge: Cambridge University Press.

VAN TIEGHEM, M. P. 1898. Avicenniacées et Symphorémacées: place de ces deux nouvelles familles dans la classification. Journal Botanique (Morot) 12: 345–352.

WAGSTAFF, S. J. and R. G. OLMSTEAD. 1997. Phylogeny of Labiatae and Verbenaceae inferred from *rbcL* sequences. Systematic Botany 22: 165–180.

WATSON, L. and M. J. DALLWITZ. 1992 onwards. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Version: 19th August 1999. http://biodiversity.uno.edu/delta/.

ZAMSKI, E. 1979. The mode of secondary growth and the three dimensional structure of the phloem in *Avicennia*. Botanical Gazette 140: 67–76.

APPENDIX 1. Taxa and Genbank accession numbers for sequences included in analyses presented here (— = no sequence available). Voucher specimens for most sequences generated by us have been reported in earlier papers: (1) McDade and Moody (1999); (2) McDade et al. (2000b); (3) McDade et al. (2000a); those reported for the first time here are followed by reference to a voucher specimen. (GB) = sequences retrieved from GenBank. The voucher of *Avicennia marina* subsp. *australasica* made by AES was lost in transit; the material came from Homebush Bay, Sydney, Australia where only this taxon of *Avicennia* occurs. Classification for Acanthaceae s.l. follows Manktelow et al. (2001).

Taxon	*rbcL*	*trnL-trnF*	nr-ITS
Acanthaceae s.l.			
Nelsonioideae			
Elytraria imbricata (Vahl) Pers.	—	AF061819 (1)	AF169852 (2)
Nelsonia campestris R.Br.	L01935 (GB)	—	—
Nelsonia canescens Spreng.	—	AF363668	—
Voucher: *Daniel et al. 5452* (CAS)			
Thunbergioideae			
Mendoncia phytocrenoides Benoist	—	AF167300 (2)	AF169849 (2)
Thunbergia alata Boj. ex Sims	—	AF061820 (1)	AF169850 (2)
T. erecta (Benth.) T. Anderson	—	AF061821 (1)	AF169851 (2)
T. mysorensis T. Anderson ex Bedd.	AY008828	—	—
Voucher: *MacDougal 5062* (MO)			
T. usambarica Lindau	L12596 (GB)	—	—
Acanthaceae s.s.			
Acanthoideae			
Acanthus mollis Graf. & Noe ex Nees	—	AF061824 (1)	—
A. montanus T. Anders.	L12592 (GB)	AF061823 (1)	—
Aphelandra boyacensis Leonard	—	AF061828 (1)	AF169759 (2)
A. campanensis Durkee	—	AF061829 (1)	AF169760 (2)
A. sinclairiana Nees	L01884 (GB)	—	—
Crossandra infundibuliformis Nees	—	AF061826 (1)	AF169754 (2)
Stenandrium pilosulum (S.F. Blake) T. F. Daniel	—	AF061827 (1)	AF169758 (2)
Ruellioideae			
Barlerieae			
Barleria lupulina Lindl.	—	AF163118 (1)	AF169751 (2)
B. prionitis L.	L01886 (GB)	—	—
B. repens Nees	—	AF063117 (1)	AF169750 (2)
Lepidagathis villosa M. Hedren	L12594 (GB)	AF063121 (1)	AF169752 (2)
Justicieae			
Dicliptera resupinata (Vahl) Juss.	—	AF063124 (1)	AF169841 (2)
Fittonia albivenis (Lindl. ex Veitch) Brummitt	—	AF289741 (3)	AF289781 (3)
Henrya insularis Nees ex Benth.	—	AF063125 (1)	AF169843 (2)
Hypoestes forskaolii (Vahl.) R.Br.	L12593 (GB)	—	—
Hypoestes phyllostachya Baker	—	AF167703 (2)	AF169842 (2)
Justicia adhatoda L.	—	AF289734 (3)	AF289773 (3)
J. americana Vahl	L14401 (GB)	—	—
J. caudata A. Gray	—	AF063134 (1)	AF169837 (2)
J. odora Lam.	L01930 (GB)	—	—
Psuederanthemum alatum (Nees) Radlk.	—	AF163130 (1)	AF169749 (2)
Razisea spicata Oerst.	—	AF063131 (1)	AF169848 (2)
Ruttya fruticosa Lindau	L02434 (GB)	AF289756 (3)	AF289801 (3)
Ruellieae			
Hygrophila corymbosa Lindau	—	AF063120 (1)	AF169836 (2)
Ruellia graecizans Backer	L12595 (GB)	—	—
R. californica (Rose) I.M. Johnst.	—	AF063115 (1)	AF167704 (2)
Sanchezia speciosa Leonard	—	AF063113 (1)	AF169385 (2)
Avicenniaceae			
Avicennia alba Blume	AY008831	AY008820	AF365980
Voucher: *Yong 86* (KE)			
A. bicolor Standl.	AY008829	AY008818	AF365977
Voucher: *Ricklefs 176* (KE)			
A. germinans (L.) L.	AY008830	AY008819	AF365979
Voucher: *Ricklefs 181* (KE)			
A. marina subsp. *australasica* (Walp.) J. Everett; Voucher: NA	AY008832	AY00821	AF365978
Bignoniaceae			
Tecoma stans (L.) Juss. ex Kunth	AF102655 (GB)	AY008826	—
Voucher for *trnL-trnF*: *Miller & Merello 8870* (MO)			

APPENDIX 1. Continued.

Taxon	*rbcL*	*trnL-trnF*	nr-ITS
Tecomaria capensis (Thunb.) Spach Voucher for *trnL-trnF*: *Holst 6056* (MO)	—	AY008827	—
Schlegelia parviflora (Oerst.) Monachino Voucher: *Gentry & Puig-Ross 14221* (MO)	L36448 (GB)	AY008825	—
Buddlejaceae			
Buddleja davidii Franch.	AJ001757 (GB)	—	—
B. marrubifolium Benth. Voucher: *Freeh & Johnson* (ARIZ)	—	AF363666	AF363671
Lamiaceae			
Ajuga reptans L.	U32163 (GB)	—	—
Callicarpa dichotoma (Lour.) K. Koch Voucher: Olmstead 88-012 (WTU)	L14393 (GB)	AF363665	—
Caryopteris bicolor (Roxb. ex Hardw.) D. J. Mabberley	U78711 (GB)	—	—
Clerodendrum speciosissimum C. Morren	—	—	U77769 (GB)
Lamium purpureum L. Voucher for *trnL-trnF*: *Wagstaff s.n.* (WTU)	Z37403 (GB)	AF363664	—
Tectona grandis L.f.	AJ001765 (GB)	—	—
Martyniaceae			
Martynia annua L.	—	AF067065 (1)	AF169854 (2)
Proboscidea louisianica (Mill.) Wooton & Standl.	L01946 (GB)	—	—
Myoporaceae			
Myoporum mauritianum DC.	L36445 (GB)	—	—
M. parvifolium R.Br. Voucher: *Starr C444* (ARIZ)	—	AF363670	—
Pedaliaceae			
Harpagophytum grandidieri Baill.	L01923 (GB)	—	—
Sesamum indicum L.	L14408 (GB)	AF067067 (1)	AF169853 (2)
Uncarina grandidieri (Baill.) Ihlenfeldt & Straka; Voucher: Olmstead 96-141 (WTU)	—	AF363667	—
''Scroph II Lineage'' of Oxelman et al. (1999)			
Antirrhinum majus L.	L11688 (GB)	—	—
Callitriche hermaphroditica L.	L36441 (GB)	—	—
Digitalis purpurea L.	X83720 (GB)	—	—
Globularia cordifolia L.	AJ001764 (GB)	—	—
Hippuris vulgaris L.	L36443 (GB)	—	—
Plantago lanceolata L.	L36454 (GB)	—	—
Veronica catenata Pennell	L36453 (GB)	—	—
Other Scrophulariaceae			
Leucophyllum frutescens (Berland.) I.M. Johnst.	AF123665	—	—
L. laevigatum Standl. Voucher: *McDade 1177* (ARIZ)	—	AF363669	—
Verbenaceae			
Bouchea fluminensis (Vell.) Moldenke	U32162 (GB)	—	—
Lantana camara L. Voucher: *Dietrich et al. 163* (MO)	—	AY008824	—
Rhaphithamnus spinosus (Juss.) Moldenke	U32160 (GB)	—	—
Stachytarpheta dichotoma (Ruiz & Pav.) Vahl Voucher for *trnL-trnF*: *Solomon 10065* (MO)	U32161 (GB)	AY008824	—
Verbena bonariensis L.	L14412 (GB)	—	—
V. urticifolia L. Voucher: *Miller et al. 8300* (MO)	—	AY008822	—
Outgroups			
Lamiales: Oleaceae			
	—	X76814 (intron) (GB)	—
Fraxinus ornis L.	—	X76822 (spacer) (GB)	—
Fraxinus velutina Torr.	—	—	AF169855 (2)
Olea europea L.	AJ001766 (GB)	—	—
Solanales: Solanaceae			
Nicotiana rustica L.	—	—	X59789 (GB)
N. tabaccum L.	—	Z00044 (GB)	—