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Phylogenetic Relationships among Acanthaceae: Evidence from Two Genomes

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ABSTRACT. We used sequence data from the nuclear ribosomal internal transcribed spacer (nr-ITS) region, both alone and in combination with data from the intron and spacer of the *trnL-trnF* chloroplast (cp) region, to study phylogenetic relationships within the large tropical and subtropical family Acanthaceae. Substitution rate in the nr-ITS region is nearly twice that of the *trnL-trnF* cp region, and more than twice the rates of other cp loci that have been sequenced for members of Acanthaceae (i.e., *rbcL, ndhF*). In terms of phylogenetic relationships, the hypothesis based on ITS was largely congruent with the *trnL-trnF* results. Exceptions are *Crossandra pungens* and the two *Acanthus* species, which are placed enigmatically by nr-ITS data. The combined analysis provides strong support for a single hypothesis of relationships among Acanthaceae sensu stricto (s.s.) and their closest relatives. 1) *Elytraria* (representing Nelsonioideae) is more distantly related to Acanthaceae s.s. than *Thunbergia* and *Mendoncia.* 2) These last two genera are sister taxa and together are the sister group of Acanthaceae s.s. 3) Acanthaceae s.s. are monophyletic. 4) There are at least four major monophyletic lineages within Acanthaceae s.s.: the *Acanthus, Barleria, Ruellia,* and *Justicia* lineages. 5) These four lineages are related as follows: {*Acanthus* lineage [*Barleria* lineage (*Justicia* + *Ruellia* lineages)]}. 6) Within the *Justicia* lineage, there are at least five monophyletic sublineages, related as follows: {*Odontonema* sublineage [*Stenostephanus* sublineage (*Henrya* sublineage {*Dicliptera* + New World *Justicia* sublineages})]}.

Acanthaceae are a large angiosperm family (ca. 3500 species, Mabberley 1997) distributed throughout tropical and subtropical regions. The plants present a rich diversity of morphological and ecological characteristics, including a wide range of floral morphologies and pollinator relationships. The family is part of Lamiales sensu lato (s.l.; i.e., sensu Olmstead et al. 1993). Based on shared presence of a fruit type that is unique among angiosperms [i.e., a few-seeded, explosively dehiscent capsule within which seeds are borne on retinaculae (the lignified derivatives of funiculae)], Acanthaceae sensu stricto (s.s., see below) have been widely accepted as a monophyletic group.

The precise delimitation of the family, however, has been controversial due to three small lineages that do not share this fruit type but seem clearly allied with Acanthaceae s.s. These are Mendoncioideae [*Mendoncia* Vell. ex. Vand. (~60 spp.) plus monotypic *Anomacanthus* Good], Thunbergioideae [*Thunbergia* Retz. (~100 spp.), *Pseudocalyx* Radlk. (~7 spp.), and monotypic *Meyenia* Nees], and Nelsonioideae (*Nelsonia* R. Br., *Elytraria* Michx., and *Staurogyne* Wall., together with ~100 species). Recently, McDade and Moody (1999) presented evidence from non-coding regions of the chloroplast (cp) genome that Mendoncioideae and Thunbergioideae are sister groups and that these together are sister to Acanthaceae s.s. The sister group relationship of these two lineages concurs with Schönenberger and Endress' (1998) results from floral structure and development. The evidence presented by McDade and Moody (1999) did not, however, unambiguously resolve relationships of Nelsonioideae. This group (represented by *Elytraria*) was not clearly more closely related to Acanthaceae s.s. plus *Mendoncia* and *Thunbergia* than it was to other relatives of Acanthaceae s.l. that were included [i.e., *Sesamum* (Pedaliaceae), *Martynia* (Martyniaceae)].

Within Acanthaceae s.s., three major lineages have been recognized by most workers, albeit at varying taxonomic levels. For example, Bentham and Hooker (1876) recognized tribes Ruellieae, Justicieae, and Acantheae; Bremekamp (1965) also recognized these three but united the first two as subfamily Ruellioideae. More recently, Scotland et al. (1994) argued for the existence of a fourth major lineage, Barlerieae, based on a unique pattern of corolla aestivation. The genera sharing this trait have been placed traditionally in disparate groups within Bremekamp's (1965) Ruellioideae (i.e., subtribe

Barleriinae of tribe Ruellieae, and tribe Lepidagathideae).

Scotland (1993), Hedrén et al. (1995), Scotland et al. (1995), and McDade and Moody (1999) have examined phylogenetic relationships among Acanthaceae. The results of these analyses are largely congruent and support the existence of four major monophyletic lineages of Acanthaceae s.s., referred to by McDade and Moody (1999) as the *Acanthus, Barleria, Ruellia,* and *Justicia* lineages. Relationships among these four lineages, however, remain incompletely understood. The *Acanthus* lineage is likely the sister group to the other three lineages because these plants lack a number of morphological characters that support monophyly of the *Barleria, Ruellia,* and *Justicia* lineages as a group (i.e., cystoliths, articulated stems, and porate pollen). However, neither morphological nor molecular evidence convincingly resolves relationships among the last three lineages. Scotland et al. (1995) proposed hygroscopic hairs on the seeds as a synapomorphy for the *Barleria* and *Ruellia* lineages. In contrast, molecular sequence data from both coding (*ndhF,* Scotland et al. 1995) and non-coding (*trnL-trnF* intron and spacer, McDade and Moody 1999) regions of the cp genome link the *Justicia* and *Ruellia* lineages as sister taxa, but without strong support.

Our goal here is to extend previous work on phylogenetic relationships among Acanthaceae s.s. and between Acanthaceae and their closest relatives using sequence data from nuclear and cpDNA genomes. Working with DNA sequence data from two genomes offers advantages including more phylogenetically informative characters, possible complementarity of evolutionary rates (and thus phylogenetic signal), and the ability to track potentially different evolutionary histories of biparental nuclear DNA and uniparental cpDNA. Here we report results based on sequences of the nuclear ribosomal internal transcribed spacer (nr-ITS) region (including ITS1, the 5.8s ribosomal gene, and ITS2; Baldwin 1992; Baldwin et al. 1995). This research was undertaken with five primary goals: (1) to explore the utility of nr-ITS compared to other loci used to date for phylogenetic work in Acanthaceae; (2) to explore further relationships among the three lineages hypothesized to be the closest relatives of Acanthaceae s.s.; (3) to test monophyly of the four major lineages within Acanthaceae s.s. proposed earlier; (4) to document relationships among these lineages; and (5) to begin to elucidate patterns of relationship within these lineages, with emphases on the *Justicia* and *Acanthus* lineages. The last three of these goals are addressed with the nr-ITS data alone and also in a combined analysis of nr-ITS and the *trnL-trnF* sequences presented earlier (McDade and Moody 1999).

Materials and Methods

Taxon Sampling. Because one of the goals of this project was to combine the nr-ITS sequences with those from the cp *trnL-trnF* intron and spacer region (McDade and Moody 1999), we attempted to obtain sequences for the same taxa that were included in that study (see Appendix in McDade and Moody 1999). Sampling density within Acanthaceae s.s. varied, with somewhat denser sampling in groups of particular interest (i.e., the *Acanthus* and *Justicia* lineages). Representatives of all three near outgroup lineages (i.e., Thunbergioideae, Mendoncioideae, Nelsonioideae) were included and we also obtained nr-ITS sequences representing Pedaliaceae (*Sesamum*) and Martyniaceae (*Martynia*) because previous analyses documented a close relationship between these and Acanthaceae s.l. (Olmstead et al. 1993; Scotland et al. 1995). *Fraxinus* was included as a more distant outgroup based on results of Wagstaff and Olmstead (1997) and Olmstead et al. (1993) that place Oleaceae near the base of Lamiales s.l. The complete sequence for *Nicotiana rustica,* retrieved from GenBank (Appendix), provided a more distant outgroup (Solanaceae, Solanales).

Molecular Methods. Fresh material was available for all but seven taxa (Appendix). Total genomic DNA was extracted using the modified CTAB method of Doyle and Doyle (1987). Some acanths have pigmented compounds that apparently complex to DNA; this was observed frequently in samples from herbarium specimens. A thorough extraction with chloroform, followed by careful washing of precipitated DNA pellets with 70% ethanol solved this problem in most cases. Occasionally, after failed attempts to amplify the nr-ITS region (see below), genomic DNAs were further purified by electrophoresing the DNA on an agarose gel, excising the band containing high molecular weight DNA, and then purifying the DNA using either dialysis or silica beads to remove agarose.

A fragment comprising ITS1, the 5.8s gene, and ITS2 (Baldwin 1992; Baldwin et al. 1995) was amplified. Early in this project, we used the ''universal'' primers ''its4'' and ''its5'' (Baldwin 1992). About 10% of the samples amplified with these primers yielded a fungal contaminant. Genbank BLAST searches matched these sequences most

TABLE 1. Changes in taxon sampling resulting from incomplete or no nr-ITS sequences for five taxa included in analysis of the cp *trnL-trnF* locus (McDade and Moody 1999). For authorities, vouchers, and Genbank accession numbers for all ITS sequences and for newly generated *trnL-trnF* sequences, see appendix.

Taxon in *trnL-trnF* analysis	Strategy for ITS analysis	Rationale
Mendoncia retusa Turrill	Substitute *M. phytocrenoides*	*M. retusa* ITS sequence extremely divergent even in ''conserved'' regions (a pseudogene?); *Mendoncia* monophyletic in *trnL-trnF* analyses
Acanthus mollis L.	Substitute *A. spinosus*	No *A. mollis* ITS sequence; *Acanthus* monophyletic in all analyses of *trnL-trnF* and ITS data
Aphelandra dolichantha Donn. Sm.	Omit	*A. dolichantha* ITS sequence incomplete; *Aphelandra* monophyletic in analyses of *trnL-trnF* and ITS data
Barleria oenotherioides Dum.-Cours.	Substitute *Lepidagathis alopecuroidea*	No *B. oenotherioides* ITS sequence; *Lepidagathis* + *Barleria* monophyletic in all analyses of *trnL-trnF* and ITS data
Peristrophe hyssopifolia Merrill	Substitute *Hypoestes phyllostachya*	*P. hyssopifolia* ITS sequence incomplete; *Peristrophe, Hypoestes, Dicliptera* monophyletic in analysis of *trnL-trnF* data

closely to fungi that are known to be both epiphyllous and endophytic (A. E. Arnold pers. comm.) suggesting that acanths have a rich array of fungal associates. Using primers ''C26A'' and ''N-nc18S10'' designed for plants (Wen and Zimmer 1996) effectively ended this problem. Optimal polymerase chain reaction (PCR) conditions to amplify double-stranded DNA varied somewhat among taxa. To circumvent the process of optimizing PCR conditions for each taxon individually, we used a ''touchdown'' temperature cycling profile. After two cycles with an initial annealing temperature of 56°C, the annealing temperature was reduced by 1°C every subsequent two cycles until 49°C was reached. Thirty cycles of amplification were then conducted at 49°C. For most specimens, this yielded a single product of ~700 nucleotides in length. If the PCR was unsuccessful or bands of other sizes were observed, we first diluted the DNA; this presumably dilutes also the impurities that inhibit PCR amplification. If problems continued, we purified the genomic DNA further as described above, and repeated the PCR reactions. PCR products were purfied with Qiagen™ Qiaquick spin-columns to remove primers and unincorporated dNTPs.

Sequences were generated on an ABI automated sequencer at the University of Arizona DNA sequencing facility using initially the same primers as in amplification. For some PCR templates, sequencing with one primer yielded sequence for the entire fragment. However, the nr-ITS region is extremely G-C rich in many Acanthaceae (see below). Three regions of ITS1 and two regions of ITS2 have a poly-C or -G string >5 bp long in most taxa. The polymerase frequently was unable to read through these long repeats of Gs or Cs such that incomplete sequences were obtained. When only a partial ITS1 sequence was obtained using primer ''N-nc18S10'' (anchored in the 18s rDNA gene), we attempted to complete the sequence using primer ''C26A'' (anchored in the 26s rDNA gene). For some templates, neither the sequencing reaction primed with ''N-nc18S10'' nor that primed with ''C26A'' yielded a complete sequence. When this occurred, we used internal primers ''its2'' and ''its3'' (Baldwin 1992), which are anchored in the 5.8s gene and yield sequence for ITS1 and ITS2, respectively. In this manner, both strands were completely or nearly completely sequenced for all but ten taxa. Sequencing with one primer yielded a complete, clean sequence for five of these ten taxa. We were ultimately unable to obtain complete sequences for the other five target taxa (i.e., those studied by McDade and Moody 1999); this resulted in minor alterations to our taxon sampling strategy (see below, Table 1).

Alignment and Analyses. Electropherograms of all sequences were proofread manually. Overlapping portions were reconciled by reverse-complementing one, aligning them, and double-checking any inconsistencies against the electropherograms. Mismataches were coded as polymorphic or as uncertain depending upon clarity of the signals. Sequences were aligned by eye in SeqApp (Gilbert 1994). Half of the total 2.2% missing data are in the highly conserved 5.8s gene. Data matrices were pre-

pared in MacClade (Maddison and Maddison 1992) and are available on request from the senior author; these were analyzed using PAUP* 4.0.0d64, provided by D. Swofford (1998), running on several Mac Power PCs and G3s. All parsimony analyses were conducted using heuristic searches with 20 random sequence addition replicates and TBR swapping; all analyses found a single island sensu Maddison (1991). Gaps were treated as missing data. To explore evidence potentially provided by indels, gaps were treated as a fifth state in an additional analysis. Multiple most parsimonious (MP) trees were combined as strict consensus trees.

The nr-ITS sequence data were also analyzed using maximum likelihood (ML) as implemented in PAUP*. Assumed nucleotide frequencies were the empirically determined values. Several ML analyses were begun with the transition:transversion ratio and rates of evolution to be estimated by the program. These analyses were stopped before completion, and the estimated transition:transversion ratio and shape parameter for the gamma distribution of rates of evolution were specified in an analysis that was completed.

The ITS region is quite divergent over the entire range of taxa included here (see below). In particular, two regions of ITS1 and one region of ITS2 were difficult to align between distant relatives. Preliminary analyses of several different alignments of these highly divergent regions yielded identical topologies and only small differences in measures of fit of the characters to the trees (results not shown). Further, it is clear that these highly variable regions are informative regarding relationships among close relatives (among which they are easily aligned). To investigate the impact of possibly faulty alignments on the results, we analyzed the data set with and without these highly variable regions.

Results from the parsimony analysis including all taxa and all data placed *Crossandra pungens* and both *Acanthus* species in unexpected phylogenetic positions (i.e., positions that are unlikely to be correct based on *trnL-trnF* sequences and morphology). The impact of these taxa on the phylogenetic outcome was explored by omitting first *C. pungens* and then both *C. pungens* and the *Acanthus* species, and reanalyzing the data following protocols described above.

In addition to standard measures of fit of characters to the resultant trees (i.e., consistency index, retention index, rescaled consistency index), the strength of support for individual branches was estimated using bootstrap values (Felsenstein 1985) and decay indices (Bremer 1988; Donoghue et al. 1992). Bootstrap (BS) values reported are from 200 ''full heuristic'' searches with 20 random sequence addition replicates and TBR branch swapping. Decay values for each branch were determined by first using MacClade (Maddison and Maddison 1992; D. Maddison pers. comm.) 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. The difference between the length of these trees and the globally shortest trees is the decay index (DI) for the branch in question.

Combined Analysis. The nr-ITS and *trnL-trnF* data sets were combined into a single NEXUS file using the file editing capabilities of PAUP. However, we were not able to achieve perfectly matched nr-ITS and *trnL-trnF* data sets because, as noted above, we were not able to obtain high quality sequences for five taxa that were included in the *trnL-trnF* study (Table 1). In four cases, the *trnL-trnF* results suggested that omitting these taxa would result in long branches (see Fig. 2 in McDade and Moody 1999). Because long branches can lead to erroneous results in parsimony analyses (Felsenstein 1978; Huelsenbeck and Hillis 1993), we substituted sequences for related taxa, obtaining both nr-ITS and new *trnL-trnF* sequences for these taxa (Table 1, Appendix). The *trnL-trnF* data were reanalyzed, using options as described above, to examine the impact of these changes in taxon sampling. This analysis yielded a topology identical to Fig. 1 in McDade and Moody (1999) (results not shown). We are thus confident that changes in taxon sampling do not confound our ability to make comparisons to the earlier analysis.

The nr-ITS and *trnL-trnF* data sets were tested for congruence using the partition homogeneity test (Farris 1995) as implemented in PAUP*. Because of the phylogenetic placement of *C. pungens* and both *Acanthus* species in the nr-ITS analysis (see below), the partition homogeneity test was conducted both with and without these taxa. Combined phylogenetic analyses were conducted as described above. Bootstrap and decay index values were generated for each branch as previously described.

Results

The nr-ITS Data. Across the phylogenetic range studied here, only conserved regions could be

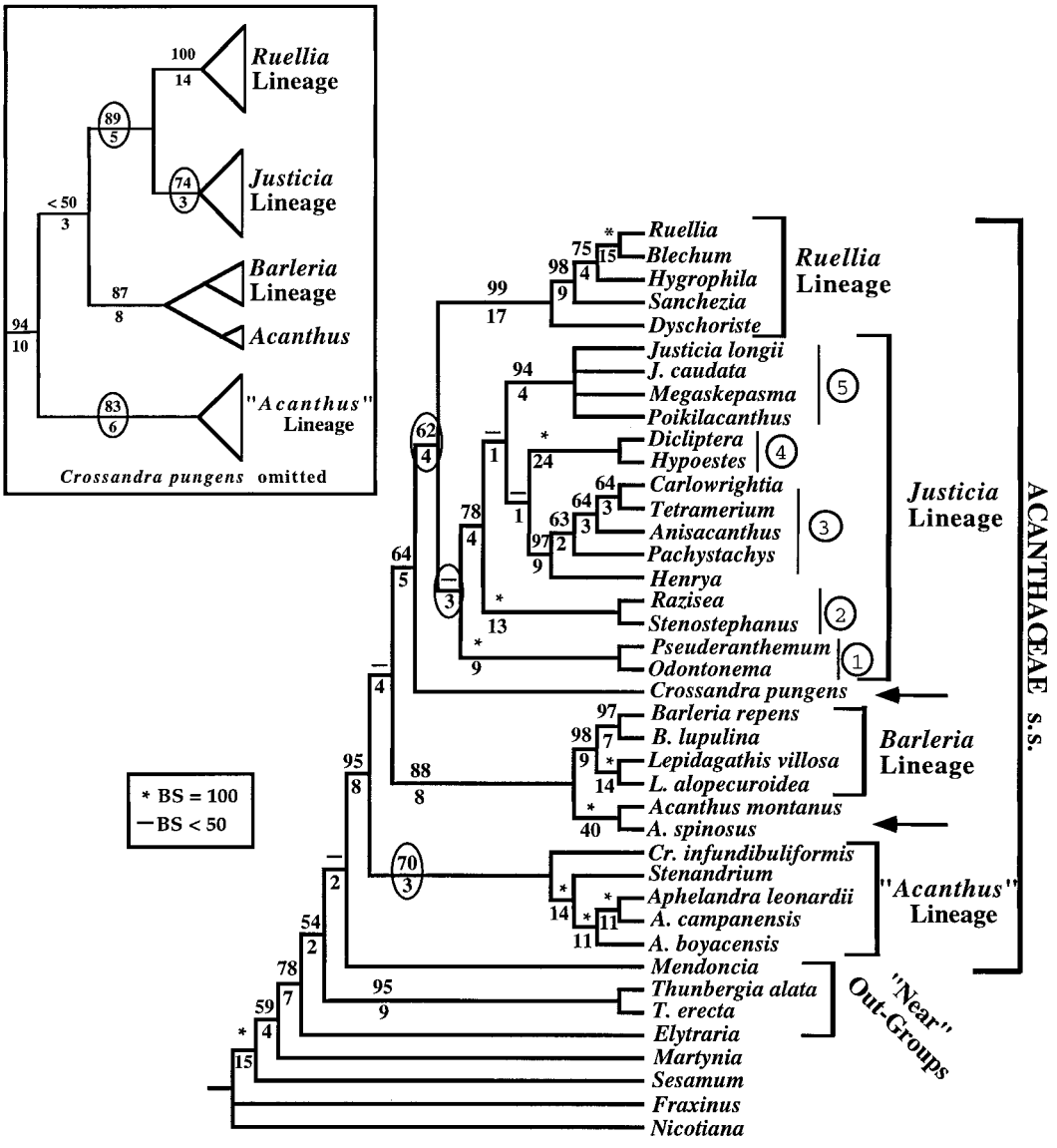

FIG. 1. Strict consensus of the three most parsimonious trees (length = 1992) from parsimony analysis of nr-ITS sequence data for all taxa (CI = 0.484, RI = 0.570, rescaled CI = 0.276). Boxed inset summarizes results of parsimony analysis omitting *Crossandra pungens* (four MP trees, length = 1952, CI = 0.490, RI = 0.572, RC = 0.280), showing only basal-most relationships. Numbers above branches are bootstrap values; those below are decay indices. Labeled groups are those referred to in the text; arrows mark enigmatically placed taxa (see text for full explanation). Circled numerals on phylogeny showing all taxa indicate sublineages within the *Justicia* lineage: (1) *Odontonema* sublineage, (2) *Stenostephanus* sublineage, (3) *Henrya* sublineage, (4) *Dicliptera* sublineage, (5) New World *Justicia* sublineage.

aligned unequivocally between distant relatives. These conserved regions included the 5.8s, as well as aligned positions 204–254 (ITS1) and 597–689 (ITS2). In less conserved regions, sequences from distant relatives could only be aligned in ''stepwise'' fashion (i.e., via phylogenetically intermediate taxa). This reflects the high rate of variation in both ITS1 and ITS2 across these taxa (i.e., proportion of variable sites and pairwise distances between taxa, Table 2). Alignment of these sequences

TABLE 2. Characteristics of the nuclear ribosomal ITS region in 38 taxa of Acanthaceae s.l. Reporting of variable and parsimony informative sites includes sites within gaps, whereas sites within gaps were excluded for calculation of pairwise distances. [1] Includes 25 and 28 bp of the 18s and 26s ribosomal genes, respectively, that flank ITS1 and ITS2, plus the 5.8s gene.

	ITS1	ITS2	nr-ITS region
Raw length	183–275	202–240	406–501
Aligned length	329	299	628 (847)[1]
Variable sites (proportion)	236 (0.72)	199 (0.67)	522 (0.62)[1]
Parsimony informative sites	175 (0.53)	137 (0.46)	376 (0.44)[1]
Pairwise distances (range, %)	0.31–26.80%	0–39.80%	0.004–26.68%
GC content, mean (range)	0.70 (0.61–0.78)	0.72 (0.61–0.79)	0.66 (0.61–0.72)

required introduction of numerous, mostly short gaps (compare raw length and aligned length in Table 2). These indels were almost exclusively in highly variable regions of the sequences. Among Acanthaceae s.l., the most divergent sequences were those of the *Lepidagathis* species, in particular *L. alopecuroidea* (this species accounts for the high value of all pairwise distance ranges, Table 2). In most taxa, ITS2 is slightly shorter and less variable than ITS1 (Table 2), but the difference is not marked. The region is extremely high in G-C content (Table 2) which presented difficulties in sequencing as described above.

Figure 1 presents the strict consensus of MP trees produced from the analysis including all taxa and characters, and treating gaps as missing data; one randomly chosen MP tree (Fig. 2) illustrates branch lengths. In terms of topology, analyses omitting the highly variable regions of ITS1 and ITS2 (i.e., those that could not be aligned across distant relatives) yielded a result that was less resolved but otherwise congruent with Fig. 1 (results not shown). This indicates that these regions contain phylogenetic information that is both congruent with and complementary to that of the more conserved regions; these results will not be discussed further.

There is moderate support for a monophyletic lineage including all three near outgroups (i.e., *Elytraria, Thunbergia* and *Mendoncia,* Fig. 1) and Acanthaceae s.s. (BS = 78, DI = 7). Relationships among these three lineages are not resolved with confidence (note BS and DI values for nodes above and below *Thunbergia*), but the two *Thunbergia* species are strongly supported as monophyletic (BS = 95, DI = 9). Acanthaceae s.s. are strongly supported as monophyletic by these data (BS = 95, DI = 8).

Within Acanthaceae s.s., there is weak support for the pattern of relationships among basal-most branches. The sister relationship between the *Justicia* and *Ruellia* lineages is not strongly supported (BS = 62, DI = 4). *Crossandra pungens* is not placed with confidence (note BS and DI values for nodes above and below this taxon), and the *Barleria* lineage (including *Acanthus*) is weakly supported as sister to *C. pungens* plus the *Justicia* + *Ruellia* lineages (BS = 64, DI = 5). The monophyly of Acanthaceae s.s. above the ''*Acanthus*'' lineage (in quotes because *Acanthus* is placed elsewhere, see below) is not strongly supported (BS < 50, DI = 4). The *Ruellia* lineage is strongly supported as monophyletic (BS = 99, DI = 17), but there is only weak support for monophyly of the *Justicia* lineage (BS = <50, DI = 3). The *Barleria* lineage is strongly supported as monophyletic but, enigmatically, this lineage includes *Acanthus* which morphology and all other analyses to date place with *Crossandra* and New World Aphelandreae (here represented by *Stenandrium* and *Aphelandra*). Finally, there is only moderate support for the ''*Acanthus*'' lineage (minus *Acanthus*) (BS = 70, DI = 3).

Sampling within the *Barleria* lineage is sparse but it is notable that both *Lepidagathis* and *Barleria* are monophyletic (Fig. 1, BS = 97, DI = 7; BS = 100, DI = 14, respectively), and that they are each others' closest relatives (BS = 98, DI = 9). Within the *Ruellia* lineage, also sparsely sampled, there is strong support for a close relationship between *Blechum* and *Ruellia* (BS = 100, DI = 15). Although both *Acanthus* species and *Crossandra pungens* are placed enigmatically by the nr-ITS data, there is support for tribe Aphelandreae [sensu Bremekamp (1965), Appendix], here represented by *Stenandrium* and three species of *Aphelandra* (BS = 100, DI = 14). Monophyly of *Aphelandra* is also strongly supported (BS = 100, DI = 11), as is that of *Acanthus* (BS = 100, DI = 40).

The *Justicia* lineage comprises all members of Bremekamp's (1965) Justicieae that were included

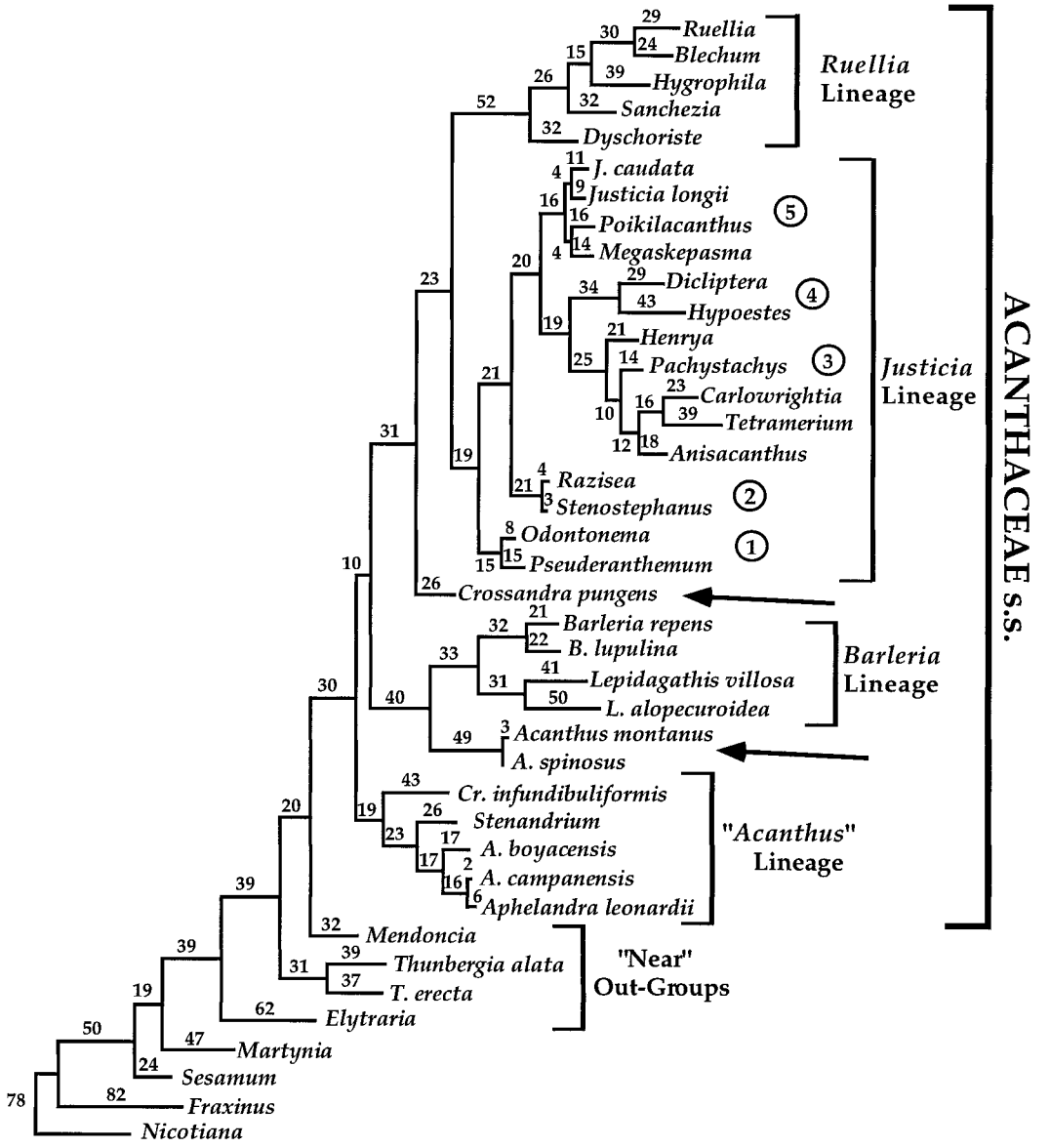

FIG. 2. One (randomly chosen) of the most parsimonious trees from the analysis of the nr-ITS sequence data for all taxa. Branch lengths are proportional to estimated number of changes using ACCTRAN optimization of PAUP*; numbers above branches are branch lengths. Labeled groups are those referred to in the text; arrows mark enigmatically placed taxa (see text for full explanation). Circled numerals indicate sublineages within the *Justicia* lineage: (1) *Odontonema* sublineage, (2) *Stenostephanus* sublineage, (3) *Henrya* sublineage, (4) *Dicliptera* sublineage, (5) New World *Justicia* sublineage.

here. Within this group, there is strong support for five monophyletic sublineages: (1) *Odontonema* sublineage (BS = 100, DI = 9), (2) *Stenostephanus* sublineage (BS = 100, DI = 13), (3) *Henrya* sublineage (BS = 97, DI = 9), (4) *Dicliptera* sublineage (BS = 100, DI = 24), and (5) New World *Justicia* and allies (BS = 94, DI = 4). The monophyly of all Justicieae above *Odontonema* + *Pseuderanthemum* is moderately

supported (BS = 78, DI = 4) but relationships among lineages of Justicieae are otherwise not clearly resolved by the nr-ITS data.

Placement of* C. pungens *and the* Acanthus *Species. In the analysis treating gaps as fifth states (results not shown), the two *Acanthus* species are placed as sister to *Crossandra infundibuliformis,* and these together are sister to *Stenandrium* + *Aphelandra. Crossandra pungens* is placed just above this lineage (i.e., basal to Acanthaceae s.s. except other members of the *Acanthus* lineage). This suggests that there is some support from indels for a traditional *Acanthus* lineage. These relationships are not, however, strongly supported (e.g., in the 50% bootstrap tree, *C. infundibuliformis* and *Acanthus* are part of an unresolved polytomy with the lineage comprised of *Stenandrium* + *Aphelandra* and that comprised of all other Acanthaceae s.s.).

From Fig. 2, it is clear that the branch supporting the two *Acanthus* species is relatively long. However, maximum likelihood analysis, which should be less sensitive to long-branch attraction than parsimony (Felsenstein 1978; Huelsenbeck and Hillis 1993), placed the two *Acanthus* species, as well as *C. pungens,* exactly as did parsimony (results not shown). This does not rule out long-branch attraction, but does suggest that this is not the only basis for placement of these taxa.

The analysis omitting *Crossandra pungens* (inset, Fig. 1) suggests that this taxon is in large part responsible for the weak support for the basal pattern of relationships (compare branches with circled BS and DI values in Fig. 1 and the inset). That is, support for monophyly of the *Justicia* lineage increases markedly (BS = 73, DI = 3), as does support for the sister group relationship between the *Ruellia* and *Justicia* lineages (BS = 89, DI = 5). In addition, removal of *C. pungens* increases support for monophyly of the ''*Acanthus*'' lineage (minus *Acanthus*) (BS = 83, DI = 6). Support for monophyly of Acanthaceae s.s. except the ''*Acanthus*'' lineage remains weak (BS = <50, DI = 3). Removal of *Acanthus* species from the analysis had little impact on phylogenetic patterns (results not shown).

Partition Homogeneity Test. All random partitions of the data yielded trees whose summed lengths exceeded the summed length of those from the original partition (i.e., the nr-ITS and cp *trnL-trnF* data sets). The lengths of the random partitions exceeded the summed value of the original by 0.4–1%. When *Crossandra pungens* was omitted from the analysis, all random partitions of the data again had summed lengths that exceeded the summed length of the original partitions, but the difference between the summed lengths of the random partitions and the original was only 0.07–0.5%. Finally, when both *Acanthus* species and *C. pungens* were removed from the analysis, 40% of random partitions had tree lengths whose sum was less than that of the original partition. That is, the null hypothesis that the nr-ITS and *trnL-trnF* data sets are homogeneous cannot be rejected. These results indicate that incongruence in the data sets stems from these three taxa which, as described above, are enigmatically placed by the ITS data.

Combined Analysis. Figure 3 presents the strict consensus of MP trees produced from the combined analysis; one randomly chosen MP tree (Fig. 4) illustrates branch lengths. The combined data strongly support monophyly of Acanthaceae s.s. plus *Mendoncia* + *Thunbergia* (BS = 94, DI = 7); these genera are sister taxa in the combined analysis with strong support (BS = 98, DI = 10). *Elytraria* is more closely related to the other Acanthaceae than to more distant relatives included (BS = 75, DI = 4). Acanthaceae s.s. are strongly supported as monophyletic (BS = 97, DI = 8), as are all four major lineages that have been identified in previous work: *Acanthus* lineage (including *Acanthus* and both species of *Crossandra*; BS = 99, DI = 15), *Barleria* lineage (without *Acanthus*; BS = 100, DI = 15), *Justicia* lineage (BS = 100, DI = 17), and *Ruellia* lineage (BS = 100, DI = 40). The *Ruellia* + *Justicia* lineages are sister groups (BS = 90, DI = 8), and the *Barleria* lineage is sister to these two together (BS = 93, DI = 7). The *Acanthus* lineage is the sister group of all other Acanthaceae s.s.

Regarding relationships within lineages, results are topologically similar to the ITS analysis with the following exceptions. As noted above, all members of Bremekamp's (1965) Acantheae + Aphelandreae are placed together as a strongly supported monophyletic *Acanthus* lineage. There is no support, however, for monophyly of the African members of this sublineage (*Crossandra* and *Acanthus*). Within the *Justicia* lineage, there is increased support for the same five sublineages supported by the nr-ITS data alone, and relationships among these sublineages are now resolved with solid support. New World *Justicia* and allies (5) are monophyletic (BS = 96, DI = 6), and they are sister to the strongly supported sister group pair *Dicliptera* + *Hypoestes* (4). These two sublineages together are sister to the *Henrya* sublineage (3) with moderate support (BS = 83, DI = 3). The *Stenostephanus* sublineage (2) is the sister group to the aforementioned three subline-

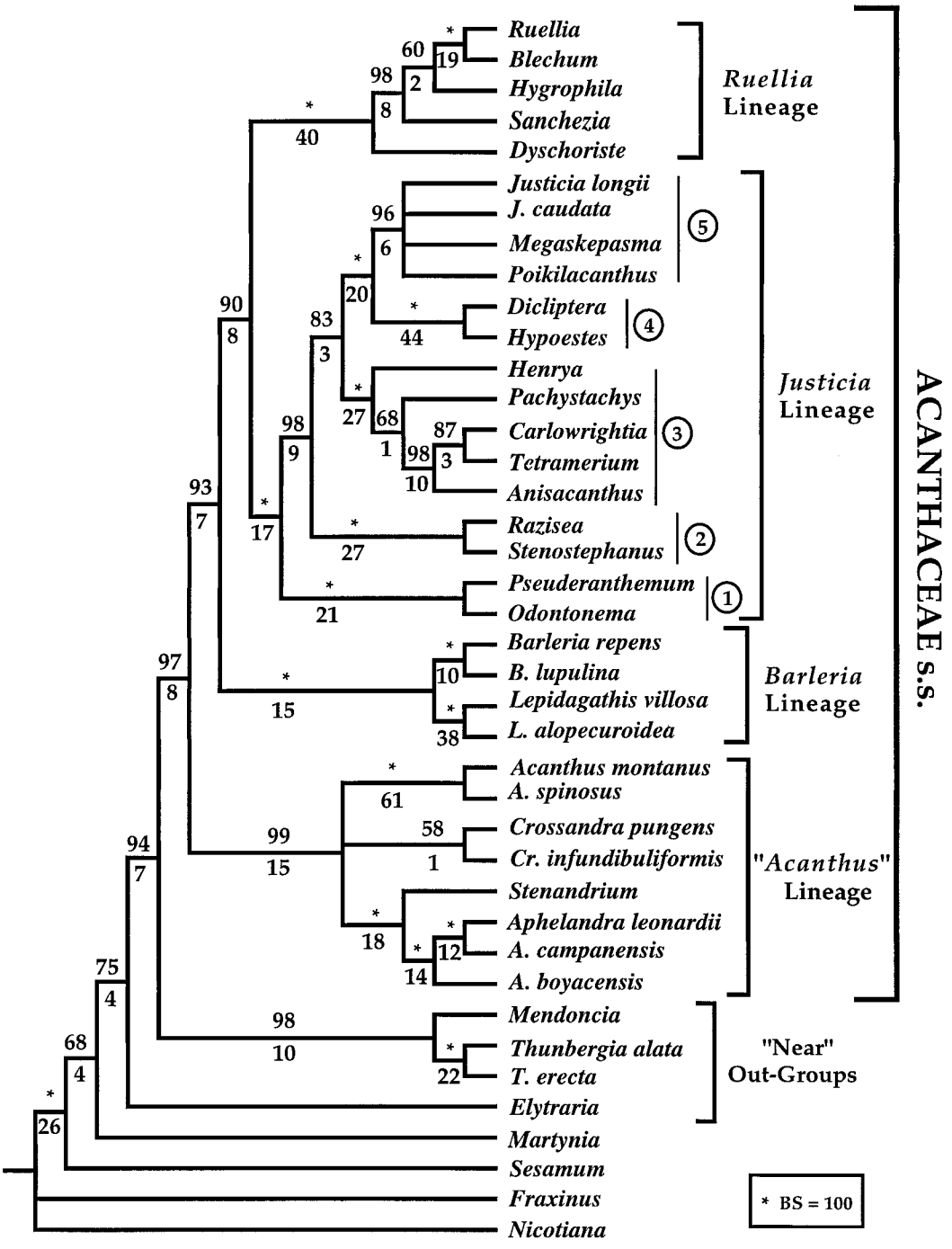

FIG. 3. Strict consensus of nine most parsimonious trees (length = 2879) from parsimony analysis of nr-ITS and cp *trnL-trnF* combined sequence data for all taxa (CI = 0.561, RI = 0.683, RC = 0.383). Numbers above branches are bootstrap values; those below are decay indices. Labeled groups are those referred to in the text. Circled numerals indicate sublineages within the *Justicia* lineage: (1) *Odontonema* sublineage, (2) *Stenostephanus* sublineage, (3) *Henrya* sublineage, (4) *Dicliptera* sublineage, (5) New World *Justicia* sublineage.

ages with strong support (BS = 98, DI = 9). Lastly, the *Odontonema* sublineage (1) is basal to the remaining Justicieae.

Discussion

Molecular Evolution. Baldwin et al. (1995) reviewed the nr-ITS locus, emphasizing patterns of evolution and phylogenetic utility. Our experience and results from nr-ITS are largely in accord with these authors' synthesis. Sequences of ITS1 in Acanthaceae are notable for their length variation [Table 2: 183–275 bp versus 187–298 bp compiled by Baldwin et al. (1995) for many plant groups], whereas those of ITS2 show less length variation [Table 2: 202–240 bp versus 187–252 bp as reported by Baldwin et al. (1995)]. The nr-ITS sequences of many plants have high G-C content as reported here, but this is not universal [e.g., Baldwin et al. (1995) report values as low as 30%]. High G-C content causes secondary structure to develop within and perhaps among PCR templates, which terminates elongation of DNA prematurely (Baldwin et al. 1995). The problems that we encountered in sequencing through regions of ITS1 and ITS2 that have poly-C or -G motifs may have been related to such secondary structures; similar problems were encountered by Downie and Katz-Downie (1996).

The relatively high rate of evolution yields regions of ITS that are difficult to align between distant relatives, a problem also noted by others (e.g., Campbell et al. 1995; Porter 1997; Downie and Katz-Downie 1996; Swensen et al. 1998). A number of approaches have been employed to handle this problem including omission of the sites (Campbell et al. 1995) and use of multiple alignments prepared with slightly different gap opening and elongation penalties (Swensen et al. 1998). We found that the highly variable regions contributed to resolution of the more distal portions of the phylogenetic hypothesis and did not conflict with or obscure phylogenetic signal from the more slowly evolving regions.

The pattern reported here of conserved regions bounded by more variable regions seems to be common among angiosperm nr-ITS sequences analyzed to date and is likely associated with the function of these molecules. Hershkovitz and Zimmer (1996) have proposed secondary structures for ITS2 based on the pattern of variable versus conserved sites [see also Fig. 3 in Baldwin et al. (1995), Fig. 1 in Buckler and Holtsford (1996)].

Several other researchers have scored indels in nr-ITS sequences as presence/absence characters or otherwise found them to be phylogenetically informative (e.g., Downie and Katz-Downie 1996; Jeandroz et al. 1997; Manos 1997). Although indels were not scored as such in the present analysis, inspection of the aligned sequences suggests that some may be informative in more narrowly circumscribed studies where alignments, and thus identification of indels, would be unambiguous. For example, members of the *Aphelandra pulcherrima* complex (sensu McDade 1984; here including *A. leonardii* and *A. campanensis*) apparently share a 30bp deletion located just before the conserved region of ITS1. Similarly, members of the *Henrya* sublineage apparently share a number of indels in this same region. Further, the parsimony analysis that treated gaps as fifth states resulted in trees congruent with the ''gaps as missing data'' analysis except that the enigmatic placement of *Crossandra pungens* and *Acanthus* was to some degree resolved. Thus, inclusion of indels as presence/absence characters may increase the phylogenetic utility of this locus in Acanthaceae.

Parsimony informative variation in the nr-ITS region (this study) is compared to that found for three chloroplast loci for Acanthaceae in Table 3 [note that the data from *rbcL* rely extensively on Hedrén et al. (1995)]. This comparison suggests that the ITS region as a whole is nearly twice as variable as the cp loci studied to date. Despite the high substitution rate, the ITS data are not markedly homoplastic as indicated by consistency indices comparable to *ndhF* and *rbcL* (Table 2). The nr-ITS data are, however, considerably more homoplastic than the *trnL-trnF* data.

Range of Utility of the nr-ITS Region. The phylogenetic range over which nr-ITS sequence data are useful in deducing patterns of relationship seems to vary among angiosperm groups. Approximately the same range of pairwise distances was documented by Downie and Katz-Downie (1996) among Apiaceae (a group similar in species richness to Acanthaceae) and by Porter (1997) for Polemoniaceae (a group with an order of magnitude fewer species than Acanthaceae). The range of pairwise distance values resulting from this study (Table 2) indicates that this locus is useful within but not among families of Lamiales s.l. That is, sequences from different families are sufficiently divergent as to present serious problems with alignment. Although this analysis represents a still sparse sample of this richly diverse family, there is evidence that the rate of evolution varies considerably among lin-

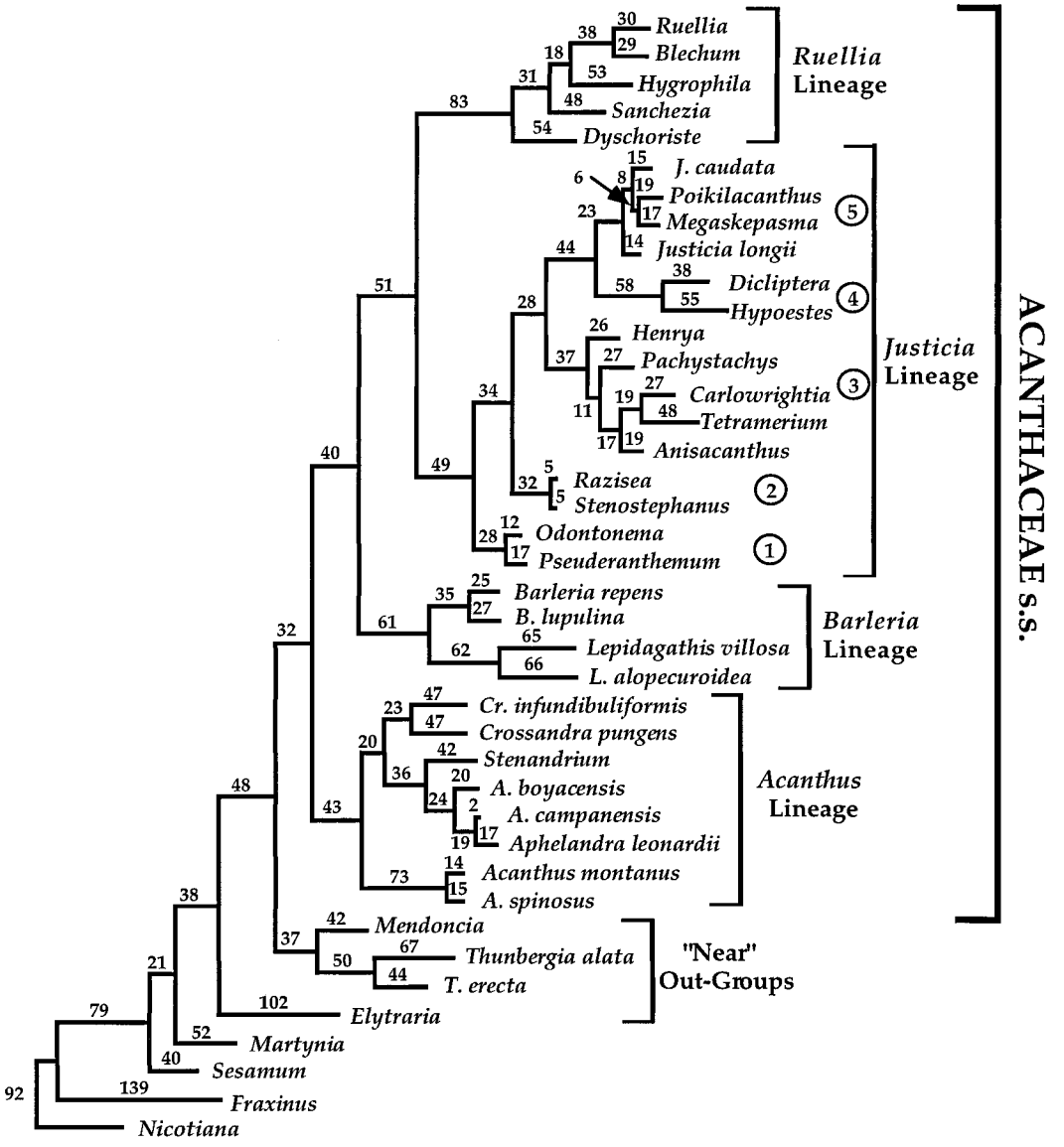

FIG. 4. One (randomly chosen) of the most parsimonious trees from the analysis of the nr-ITS and cp *trnL-trnF* combined sequence data for all taxa. Branch lengths are proportional to estimated number of changes using ACCTRAN optimization of PAUP*; numbers above branches are branch lengths. Labeled groups are those referred to in the text. Circled numerals indicate sublineages within the *Justicia* lineage: (1) *Odontonema* sublineage, (2) *Stenostephanus* sublineage, (3) *Henrya* sublineage, (4) *Dicliptera* sublineage, (5) New World *Justicia* sublineage.

eages. For example, the four sampled members of New World *Justicia* and allies are not likely to be closely related based on morphology or taxonomy. The group includes at least 400 species and is richly diverse morphologically: among species in this lineage there are trees, shrubs and herbs, and corolla length ranges from a few mm to at least 8 cm. There is almost no sequence variation among the four taxa included suggesting a rapid radiation of this lineage. In contrast, *Barleria* and *Lepidagathis* represent a group of similar size within which there is comparable morphological diversity but also much sequence variation. The nr-ITS region is not promising as a phylogenetic tool among New

TABLE 3. Comparison of three chloroplast regions to the nuclear ribosomal ITS [data for nr-ITS from this study (Table 2), *trnL-trnF* from McDade and Moody (1999), *ndhF* and *rbcL* from Scotland et al. (1995)]. Caution is warranted in drawing conclusions from this comparison because this study and that of McDade and Moody (1999) included more than twice as many taxa as Scotland et al. (1995), although representing the same phylogenetic range. Rate is frequency per aligned base; consistency indices exclude autapomorphies. Indels were not scored in the present analysis of nr-ITS sequences, although they are likely to be informative in analyses among more closely related taxa (see text for full explanation).

	nr-ITS	*trnL-trnF*	*ndhF*	*rbcL*
Aligned length	847	1152	2223	1428
Parsimony informative variation				
Substitutions	376	263	421	136
Rate	0.44	0.23	0.19	0.09
Length Mutations	—	54	6	0
Rate	—	0.047	0.003	0
Consistency Index	0.49	0.75	0.53	0.37

World *Justicia* and allies, but clearly is so for members of the *Barleria* lineage.

Phylogenetic Relationships of Acanthaceae. The separate analyses of nr-ITS (this paper) and *trnL-trnF* sequence data (McDade and Moody 1999) differ only in weakly supported portions of the phylogeny, except for the placement of *Acanthus.* The enigmatic placement of *C. pungens* and *Acanthus* by the nr-ITS data defies facile explanation. The placement of *C. pungens* is suggestive of hybrid origin; McDade (1992) showed that many hybrids are placed by phylogenetic analysis between the two parents and as the basal member of the clade that includes the most derived parent. Thus, in this case, *C. pungens* would be supposed to have one parent that is a member of the ''*Acanthus*'' lineage, with the other, more derived, parent being a member of either the *Ruellia* or the *Justicia* lineage. This notion is unlikely, however, given the taxonomic level involved here: the putative parents would be extremely distantly related plants. In any event, support for any placement of *C. pungens* is weak. In contrast, the nr-ITS data place *Acanthus* quite robustly (Fig. 1). The maximum likelihood analysis gave essentially identical results, suggesting that the problem is not simply one of long-branch attraction (Felsenstein 1978; Huelsenbeck and Hillis 1993). That the parsimony analysis treating gaps as fifth characters brought these taxa closer to other members of the ''*Acanthus*'' lineage suggests that reexamination of indels as potentially informative characters is warranted. In neither case, however, are the nr-ITS data sufficient to outweigh the *trnL-trnF* data which strongly support placement of these taxa with other members of the *Acanthus* lineage.

The combined analysis apparently brought together and reinforced the more strongly supported components of the separate analyses, resulting in a single hypothesis of relationships among Acanthaceae and their closest relatives (Fig. 3). The most significant phylogenetic patterns that emerge are as follows:

(1) *Elytraria* (representing Nelsonioideae) is more closely related to other Acanthaceae s.l. than to more distant relatives included in the analysis. The ITS data alone support this pattern of relationships, whereas the *trnL-trnF* data were unable to resolve relationships at this level. The results of Hedrén et al. (1995) and Scotland et al. (1995) concur with the combined analysis presented here, placing Nelsonioideae as sister to other Acanthaceae.

(2) *Mendoncia* and *Thunbergia* are one another's closest relatives; these together are sister to Acanthaceae s.s. The *trnL-trnF* analysis (McDade and Moody 1999) supports these patterns whereas the nr-ITS data contradict them only weakly (BS < 50 for node placing *Mendoncia* above *Thunbergia,* Fig. 1). Floral anatomical data also support a close relationship between *Thunbergia* and *Mendoncia* (Schönenberger and Endress 1998), and these plants share an ''epicalyx'' of paired bracts subtending the flowers, highly modified rudimentary calyx, and sprawling or climbing habit (Bremekamp 1965; L. A. McDade pers. obs.). That these taxa have a closer relationship to Acanthaceae s.s. than Nelsonioideae is supported by shared loss of endosperm and reduction in number of ovules. *Mendoncia* was not included by Hedrén et al. (1995) or Scotland et al. (1995), but both placed *Thunbergia* as either basal to a monophyletic Acanthaceae s.s. (*ndhF*) or as part

of a polytomy with lineages belonging to Acanthaceae s.s. (*rbcL*).

(3) Acanthaceae s.s. are strongly supported as monophyletic. As described above, all Acanthaceae s.s. share at least one unique and unreversed morphological synapomorphy: retinaculae subtending the seeds. It was thus surprising that the *trnL-trnF* data alone did not support monophyly of Acanthaceae s.s. (McDade and Moody 1999). However, the cp data did not strongly support an alternative hypothesis and, combined with strong support for monophyly of Acanthaceae s.s. from the nr-ITS data, the result is congruent with morphology.

(4) There are four major monophyletic lineages within Acanthaceae s.s., in accord with previous phylogenetic work (Scotland et al. 1995; McDade and Moody 1999). Notably, our results confirm the ideas of Scotland et al. (1994) regarding distinctiveness of the *Barleria* lineage from the *Ruellia* lineage. The only discord is with regard to some members of the *Acanthus* lineage in the nr-ITS analysis, as described above. The *trnL-trnF* data strongly support monophyly of the Old World members of the *Acanthus* lineage [Bremekamp's (1965) Acantheae, here represented by *Crossandra* + *Acanthus,* see Fig. 1 in McDade and Moody 1999]. In contrast, these are not monophyletic in the combined analysis, reflecting discord between the nr-ITS and *trnL-trnF* data sets. Interestingly, monophyly of the Acantheae is supported by morphology (the upper lip of the corolla is deeply slit in *Crossandra* and virtually lacking in *Acanthus*), whereas no unique morphological synapomorphies have yet been identified for Aphelandreae (here represented by *Stenandrium* and *Aphelandra*). In fact, Vollesen (1992) has proposed transfer of *Stenandrium* from Aphelandreae to Acantheae. Our results do not support this change but sequences for more species, including Old World *Stenandrium,* are necessary to test generic and tribal delimitations. These results address a number of other controversies regarding assignment of some taxa to these four lineages as reported earlier by McDade and Moody (1999); these are not reiterated here.

(5) The combined data provide strong support for one hypothesis of relationships among the four lineages of Acanthaceae s.s.: {*Acanthus* lineage [*Barleria* lineage (*Justicia* + *Ruellia* lineages)]}. In contrast, neither the *trnL-trnF* nor the nr-ITS data alone were able to resolve relationships among these lineages. The result from the combined analysis is at least partly supported by morphology in that a number of characters support monophyly of the *Barleria, Ruellia,* and *Justicia* lineages as a group: presence of cystoliths, articulated stems, and porate pollen. We know of no morphological support for the relationship between the *Justicia* and *Ruellia* lineages, but the analysis of Scotland et al. (1995) based on sequence data for the cp *ndhF* gene also supports this relationship.

(6) Within the *Justicia* lineage, the nr-ITS data alone do not resolve relationships among the five sublineages (Fig. 1). In contrast, the *trnL-trnF* data strongly support the same pattern of relationships that emerges from the combined analysis (Fig. 3). McDade and Moody (1999) discussed sublineages of the *Justicia* lineage, including morphological support and likely membership beyond the taxa included here. Because Old World members of *Justicia* are not included in the present analysis, it is premature to assume that the five recognized sublineages will accommodate all of the diversity in the lineage as a whole. On-going research will document further the relationships among members of the extremely diverse (ca. 2000 species) *Justicia* lineage using additional taxa, in particular, more Old World representatives. This work is using the same molecular loci studied here, as well as evidence from morphology and chromosomes.

In sum, the combined analysis yielded a single strongly supported hypothesis regarding relationships among major lineages of Acanthaceae s.s., and between these and their closest relatives (Fig. 3). Against this framework, it should be feasible to determine the phylogenetic placement of enigmatic lineages of Acanthaceae (e.g., Whitfieldieae, M. Manktelow pers. comm.), as well as to explore the relationships of additional members of Lamiales s.l. that are likely close to Acanthaceae. It is important to note that a great deal remains to be achieved in terms of understanding phylogenetic relationships within major lineages. Each of these is richly diverse morphologically and each is essentially worldwide in distribution. As a result, progress within major lineages will be necessary to understand morphological evolution and biogeography of Acanthaceae.

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APPENDIX 1.

Taxa, GenBank accession number, and sources of plant materials from which DNA was extracted for sequencing of the nuclear ribosomal internal transcribed spacer region. Fresh material was used except as indicated (HS = herbarium specimen; S = silica dried). Sequence for *Nicotiana rustica* was retrieved from GenBank. Classification follows Bremekamp (1965) except that Nelsonioideae (excluded by Bremekamp from Acanthaceae) are here treated as a subfamily; Bremekamp's classification is the most recent comprehensive work.

Nelsonioideae: *Elytraria imbricata* (Vahl) Pers.; AF169852; Arizona, Santa Cruz County, Flux Canyon SW of Patagonia, *McDade & Jenkins 1155* (ARIZ).

Thunbergioideae: *Thunbergia alata* Boj. ex Sims; AF169850; Cultivated from commercial seed, Thompson and Morgan. *T. erecta* (Benth.) T. Anders.; AF169851; Cultivated, Missouri Botanical Garden, Accession No. 802421.

Mendoncioideae: *Mendoncia phytocrenoides* Benoist; AF169849; Cultivated, Zurich Botanical Garden, Switzerland (BGZ 19981162) (S); *trnL-trnF* sequence also generated for this analysis: AF167330.

Acanthoideae

Acantheae: *Acanthus montanus* T. Anders.; AF169756; Cultivated, Duke University greenhouses, Accession No. 86–169. *A. spinosus* L.; AF169757; Cultivated, Strybing Arboretum, San Francisco, *Anderson 3696* (CAS) (S); *trnL-trnF* sequence also generated for this analysis: AF1673301. *Crossandra infundibuliformis* Nees; AF169754; Cultivated, U. Arizona, *McDade 1162* (ARIZ). *C. pungens* Lindau; AF169755; Cultivated, Conservatory, Golden Gate Park, San Francisco (CAS 959662), *Daniel s.n.* (S).

Aphelandreae: *Stenandrium pilosulum* (Blake) T. F. Daniel; AF169758; Mexico, Sonora State, Yécora Municipio, El Kipor, *Van Devender & Reina G. 97–434* (ARIZ). *Aphelandra boyacensis* Leonard; AF169759; Colombia, Antioquia Province, Río Claro, El Refugio, *McDade 989* (DUKE). *A. campanensis* Durkee; AF169760; Panama, San Blas Province, near Mandinga, Río Mandinga, *McDade 852* (DUKE). *A. leonardii* McDade; AF169761; Costa Rica, San José Province, Frailes, *McDade 310* (DUKE).

Ruellioideae

Trichanthereae: *Sanchezia speciosa* Leonard; AF169835; Cultivated, Duke University greenhouses, Accession No. 66–462.

Ruellieae

Blechinae: *Blechum pyramidatum* (Lam.) Urb.; AF167705; Costa Rica, Puntarenas Province, Wilson Botanical Garden, *McDade 441* (DUKE).

Ruelliinae: *Ruellia californica* (Rose) I. M. Johnst.; AF167704; Cultivated, U. Arizona campus, *McDade 1157* (ARIZ).

Barleriinae: *Barleria lupulina* Lindl.; AF169751; Cultivated, Conservatory, Golden Gate Park, San Francisco (CAS 952700), *Daniel s.n.* (S). *B. repens* Nees; AF169750; Cultivated, Missouri Botanical Garden, Accession No. 97003.

Petalidiinae: *Dyschoriste decumbens* (A. Gray) Kuntze; AF169834; Arizona, Santa Cruz County, Canelo Hills, *McDade & Jenkins 1156* (ARIZ).

Hygrophilinae: *Hygrophila corymbosa* Lindau; AF169836; Cultivated, Missouri Botanical Garden, Accession No. 897223.

Lepidagathidae: *Lepidagathis alopecuroidea* (Vahl) R. Br.; AF169753; Panama, Panama Province, summit of Cerro Jefe, *Daniel et al. 8066* (CAS); *trnL-trnF* sequence also generated for this analysis: AF167702. *L. villosa* Hedrén; AF169752; Scotland et al. 1995 [DNA provided by R. Olmstead (University of Washington) and R. Scotland (Oxford University)].

Justicieae

Odontoneminae: *Anisacanthus thurberi* (Torr.) A. Gray; AF169846; Arizona, Pima County, Tucson Mountains, *Van Devender 88–150* (ARIZ). *Carlowrightia arizonica* A. Gray; AF169845; Arizona, Pima County, Tucson Mountains, *Jenkins 89–24* (ARIZ). *Dicliptera resupinata* (Vahl) Juss.; AF169841; Arizona, Pima County, Santa Catalina Mountains, *Van Devender 84–269* (ARIZ). *Henrya insularis* Nees ex Benth.; AF169843; Mexico, Sonora State, near Alamos, *Jenkins 89–432* (ARIZ). *Hypoestes phyllostachya* Baker; AF169842; Cultivated, U. Arizona, *McDade 1232* (ARIZ); *trnL-trnF* sequence generated for this analysis: AF167703. *Megaskepasma erythrochlamys* Lindau; AF169840; Costa Rica, Puntarenas Province, Wilson Botanical Garden, *McDade 253* (DUKE). *Odontonema tubiforme* (Bertol.) Kuntze; AF169748; Cultivated, Duke University greenhouses, Accession No. 66–153. *Pachystachys lutea* Nees; AF169844; Cultivated, Duke University greenhouses, Accession No. 84–055. *Pseuderanthemum alatum* (Nees) Radlk.; AF169749; Cultivated, Duke University greenhouses, Accession No. 84–055. *Razisea spicata* Oerst.; AF169848; Costa Rica, Heredia Province, La Selva Biological Station, *Hammel 7974* (DUKE). *Stenostephanus silvaticus* (Nees) T. F. Daniel; AF169747; Costa Rica, San José Province, Parque Nacional Braulio Carrillo, *Maas 7800* (MO) (HS).

Justiciinae: *Justicia caudata* A. Gray; AF169837; Mexico, Sonora State, near Alamos, *Faivre 64* (ARIZ). *J. longii* Hilsenb.; AF169839; Arizona, Pima County, Tucson Mountains, *Van Devender 87–307* (ARIZ). *Poikilacanthus macranthus* Lindau; AF169838; Costa Rica, Alajuela Province, Monteverde Reserve, *Haber 707* (MO) (HS).

Martyniaceae: *Martynia annua* L.; AF169854; Mexico, Sonora State, Municipio de Alamos between Sabanito Sur and Alamos, *Jenkins 97–149* (ARIZ).

Pedaliaceae: *Sesamum indicum* L.; AF169853; Mexico, Sonora State, Municipio de Alamos between Navojoa and Alamos, *Jenkins 97–141* (ARIZ).

Oleaceae: *Fraxinus velutina* Torr.; AF169855; Cultivated, U. Arizona Campus, *McDade 1235* (ARIZ).

Solanaceae: *Nicotiana rustica* L.; X59789