## PHYLOGENETIC RELATIONSHIPS WITHIN THE TRIBE JUSTICIEAE (ACANTHACEAE): EVIDENCE FROM MOLECULAR SEQUENCES, MORPHOLOGY, AND CYTOLOGY[1]

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### ABSTRACT

We used molecular sequence data from the nuclear ribosomal internal transcribed spacers and from the intron and spacer of the *trn*L-*trn*F chloroplast region to study phylogenetic relationships within the large (ca. 2000 species), wide-ranging, and taxonomically difficult tribe Justicieae (Acanthaceae). The partition homogeneity test indicated that the data sets for the two loci were congruent, and separate analyses of the two gave similar results. Analysis of the combined data set provides a highly resolved hypothesis of relationships, much of it strongly supported. Justicieae are strongly supported as monophyletic; within the tribe, five lineages and one paraphyletic grade are related as follows: [*Pseuderanthemum* lineage (Isoglossinae {*Tetramerium* lineage [multiple clades of Old World "justicioids" (Diclipterinae + New World "justicioids")]})]. Many aspects of this phylogenetic hypothesis are supported by data from morphology and cytology, and some conform to earlier classifications of the group. There are, however, a number of novel aspects. Notably, the large genus *Justicia* (ca. 700 species) is not monophyletic; the Old World members form a grade and the New World members are monophyletic only if a number of other genera are included. The very strongly supported sister-group relationship between Diclipterinae and the New World "justicioid" lineage is novel, and we cannot identify non-molecular synapomorphies to confirm this relationship. *Rhinacanthus*, a "justicioid" (*Justicia* and morphologically similar genera) by all but phylogenetic criteria, is strongly supported as a basal member of Diclipterinae, and cytological evidence confirms this placement. The *Pseuderanthemum* lineage is only modestly supported as monophyletic and may, in fact, represent a series of basal lineages. These plants are marked by having four staminal elements (four stamens or two stamens plus two staminodes), a plesiomorphic condition for all Acanthaceae. Additional evidence (both taxa and characters) will be necessary to resolve this uncertainty, as well as to determine the phylogenetic status of Old World "justicioids." Our analysis does provide considerable resolution of relationships within monophyletic lineages, and these relationships are discussed in the context of non-molecular evidence and previous classifications.

*Key words:* Acanthaceae, cp *trn*L-*trn*F, cytology, Justicieae, Lamiales, molecular sequences, morphology, nr ITS, phylogenetics.

"I will not say that . . . [my] classification of the Justicieae . . . is perfect. On the contrary, I am convinced that it is no more than a tentative effort" (Bremekamp, 1965: 30).

The tribe Justicieae is the largest and arguably most systematically challenging lineage in the plant family Acanthaceae. Justicieae comprise about 2000 species, and their nearly worldwide distribution essentially matches that of the family as a whole. As documented by Lindau (in 1895) and supplemented by numerous other contributions since that time, the macromorphological (especially habit and floral) and palynological diversity within the tribe is considerable. Composition, infratribal relationships, and affinities of Justicieae have been controversial ever since Lindau's (1895) comprehensive infrafamilial classification of Acanthaceae. Lindau grouped *Justicia* L. and several of its presumed close relatives into a tribe characterized by having an androecium of two stamens and "Knötchenpollen" (2- or 3-aperturate pollen with 1 to 3 rows of insulae on each side of the apertures). The fact that several taxa of Lindau's Justicieae do not have this type of pollen whereas several genera with two stamens and "Knötchenpollen" were classified in other tribes by Lindau is indicative of problems with his classification.

Tribe Justicieae was classified by Lindau as part of "Imbricatae," a supratribal group that was defined by imbricate corolla aestivation. This pattern of aestivation is likely primitive for Acanthaceae, and Imbricatae are a heterogeneous group, as has been recognized for some time (Bremekamp, 1965) and confirmed by recent phylogenetic work (Hedrén et al., 1995; Scotland et al., 1995; McDade & Moody, 1999; McDade et al., 2000). Further, Scotland et al. (1994) showed that there is more than one variant of imbricate aestivation among Acanthaceae. Other authors have demonstrated that aestivation varies within lineages (Manktelow et al., in press) and even within genera (Schönenberger & Endress, 1998) such that its use as a defining character may be unwarranted. Moreover, Lindau's (1895) tribes and subtribes of Imbricatae, including Justicieae as described above, are not clearly circumscribed morphologically, and many are disparate assemblages. The character basis for the classification is inconsistent in that characters used by Lindau in his keys sometimes conflict with those employed in descriptions of tribes or genera included therein. For example, Isoglossinae are characterized as having two stamens, but plants of some genera placed in this subtribe by Lindau have four. Many of Lindau's taxa contain a core of genera that undoubtedly belong together, along with an odd assemblage of others. For example, his Diclipterinae include *Dicliptera* Juss., *Hypoestes* Sol. ex R. Br., *Periestes* Baill. (= *Hypoestes*), and *Peristrophe* Nees, a group readily recognized as cohesive by essentially all students of Acanthaceae. To this subtribe, however, Lindau added *Tetramerium* Nees. Plants of this last genus are morphologically more similar to *Carlowrightia* A. Gray and *Anisacanthus* Nees, which were placed by Lindau in Graptophylleae, than they are to other Diclipterinae. These problems have made it difficult for subsequent students of Acanthaceae to classify newly described genera with any confidence, such that Lindau's classification is not widely used.

Bremekamp (1965) outlined a revised infrafamilial classification of Acanthaceae, but did not always specify the generic composition of his tribes and subtribes. In a major realignment of taxa, he included six of the tribes placed by Lindau in Imbricatae (Asystasieae, Graptophylleae, Isoglosseae, Justicieae, Odontonemeae, Pseuderanthemeae) into an expanded Justicieae with three subtribes (Justiciinae, Rhytiglossinae, and Odontoneminae). Justiciinae and Rhytiglossinae (a substitute name for Lindau's Isoglossinae) were distinguished from Odontoneminae on the basis of an androecium of two stamens with no staminodes. Justiciinae were further distinguished by presence of a rugula (a channel in the upper lip of the corolla in which the style rests during anthesis), whereas plants with lenticular biporate pollen were assigned to Rhytiglossinae (= Isoglossinae). Most of the remaining Justicieae were placed in Odontoneminae. Plants belonging to this last group retain the plesiomorphic state of one or more of the characters that marked

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Bremekamp's other two subtribes, including absence of a rugula, androecium with four staminal elements (four fertile stamens or two stamens plus two staminodes), and pollen with colpoid streaks (= pseudocolpi). It is mostly feasible to assign newly described genera to Bremekamp's subtribes; however, Justiciinae and Odontoneminae, in particular, are very large groups of plants such that little is really accomplished by classifying to this level. Further, it is clear that although Justiciinae and Rhytiglossinae (= Isoglossinae) are likely marked by synapomorphies and may be monophyletic, Odontoneminae are not and are thus likely not monophyletic.

Problems in classifying Justicieae sensu Bremekamp (and as used herein) do not end at the subtribal level. The tribe includes a number of genera that are not clearly diagnosed as distinct from related genera (including *Justicia* with ca. 700 species; see Graham, 1988, who noted a conservative estimate of some 600 species at that time). This often makes routine identification difficult at best. In sum, there is a great deal to be learned about relationships in this richly diverse lineage of Acanthaceae.

Recent progress has affirmed the monophyletic status of Acanthaceae and advanced our understanding of relationships between Justicieae and the three other main lineages of Acanthaceae (McDade et al., 2000). Justicieae and Ruellieae sensu lato (s.l., i.e., sensu Manktelow et al., in press) are sister taxa. Barlerieae (including Whitfieldieae, see Manktelow et al., in press) are sister to these two together, and Acanthoideae (sensu Bremekamp, 1965) are sister to the first three together. The monophyly of each of these, as well as the pattern of relationships among them, is supported by molecular sequence data and, in some cases, by morphological synapomorphies (McDade & Moody, 1999; Manktelow et al., in press). There has, however, been little progress in understanding relationships within Justicieae. Based on admittedly sparse sampling, particularly of Old World plants, McDade and Moody (1999) and McDade et al. (2000) proposed at least five sublineages of Justicieae. Other authors, notably Daniel (1986), Daniel and Chuang (1993), and Daniel et al. (1984, 1990), have informally proposed that certain groups of genera are closely related, but these proposals have not been in the context of phylogenetic analyses nor have they been formalized.

Here we present a phylogenetic analysis based on molecular sequence data from two regions (the nuclear ribosomal ITS and chloroplast *trn*L-*trn*F spacer and intron) for a reasonably representative sample of Justicieae. We discuss these results in the context of evidence from other sources including morphology, chromosome numbers, and geographic distribution.

## MATERIALS AND METHODS

### TAXON SAMPLING

We obtained sequences for species representing all of Lindau's (1895) suprageneric taxa of Imbricatae that were included by Bremekamp (1965) in his Justicieae. In addition, we attempted to sample two or more species of the larger genera of Justicieae, with emphasis on genera that occur in both the Old and New Worlds, e.g., *Justicia, Dicliptera*. From the very large genus *Justicia*, we included representatives of more than half of the sections recognized by Graham (1988). To root our hypothesis of relationships among Justicieae, we used two members of each of the other main lineages of Acanthaceae s. str. as outgroups: Ruellieae s.l. (one species each of *Ruellia* and *Sanchezia*), Barlerieae (one species each of *Barleria* and *Lepidagathis*), and Acanthoideae (one species each of *Aphelandra* and *Stenandrium*) (Appendix 1). We included the most distantly related groups within these lineages for which we had material and were able to obtain sequences. This should have the effect of "breaking" long branches (sensu Felsenstein, 1985) and thus increase confidence in our results (see Graybeal, 1998).

### MOLECULAR METHODS

For most samples, DNA was extracted from fresh leaf material or material dried in silica gel; recently collected herbarium specimens were the source of DNA for the remaining species (Appendix 1). 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 dealt with by purifying the DNA, as described by McDade et al. (2000).

For the nr ITS sequences, a fragment comprising *its*1, the 5.8s gene, and *its*2 (Baldwin, 1992; Baldwin et al., 1995) was amplified. Early in this project, we used the "universal" primers "its4" and "its5" (Baldwin, 1992). As noted by McDade et al. (2000), some samples amplified with these primers yielded fungal contaminants. Using primers "C26A" and "N-nc18S10" designed for plants (Wen & Zimmer, 1996) effectively ended this problem. Optimal polymerase chain reaction (PCR) conditions to amplify double-stranded DNA varied somewhat among taxa, and we used a "touchdown" tem-

perature cycling profile to circumvent the process of optimizing PCR conditions for each taxon (see McDade et al., 2000). For the *trn*L-*trn*F sequences, a fragment comprising the *trn*L intron, the 3'*trn*L exon, and the intergenic spacer between this exon and the *trn*F gene of the chloroplast genome (Taberlet et al., 1991) was amplified using the "c" and "f" primers designed by these same authors. Standard PCR techniques were used to amplify double-stranded DNA.

Sequences were generated on ABI automated sequencers at the University of Arizona DNA sequencing facility using initially the same primers as in amplification. This yielded high quality sequences for essentially all of the cp *trn*L-*trn*F PCR templates and for about two-thirds of the nr ITS samples. However, the nr ITS region is extremely G-C rich in many Acanthaceae (see McDade et al., 2000) and poly-C or -G strings > 5 bp long are present in some taxa. DNA polymerase frequently was unable to read through these long repeats of Gs or Cs such that incomplete sequences were obtained. When only a partial *its*1 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 sequenced using internal primers "its2" and "its3" (Baldwin, 1992), which are anchored in the 5.8s gene and yield sequence for *its*1 and *its*2, respectively. Both strands were sequenced except when sequencing with one primer yielded a complete sequence with no ambiguities (ca. ½ of the cp *trn*L-*trn*F templates and ¼ of nr ITS templates) or when sequencing with all available primers did not yield completely overlapping forward and reverse sequences (five of the nr ITS templates).

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 ANALYSES

Sequences were aligned separately by eye in SeqApp (Gilbert, 1994), and then moved into MacClade version 4.0a11 (Maddison & Maddison, 1999). These are available on request from LAM. As documented by McDade et al. (2000), the nr ITS region is quite divergent among Acanthaceae.

Although alignments were straightforward within Justicieae, one region of *its*1 (aligned positions 65–204; ca. 15% of the total aligned length) could not be aligned with confidence between Justicieae and the six taxa used as outgroups. The six outgroup taxa were thus scored as missing for those nucleotide positions. Most of the total 3.5% missing data for the ingroup are in the highly conserved 5.8s gene. Most of the remaining missing data are in the *its*1 sequences for three taxa (*Fittonia* Coem., *Asystasia gangetica*, and *Spathacanthus parviflorus*); these sequences could not be completed despite attempts using all available primers. Alignment of the nr ITS sequences required introduction of many gaps, most of which were one or two bp in length, restricted to a single taxon, and in highly variable and/or G-C rich regions of the sequences. However, 14 indels were shared by two or more taxa, had concordant 5' and 3' termini, and did not overlap gaps in the sequences for other taxa. Information on these indels was added to the matrix as presence/absence characters.

The cp sequences were easily aligned across all taxa, including outgroups, despite the fact that, as noted by McDade and Moody (1999), this region is prone to length mutations. Seventeen parsimony informative indels were added to the matrix as presence/absence data. For the cp locus, 3.2% of data are missing; almost all of the missing data are in the relatively conserved 5' end of the intron.

Data matrices were analyzed separately using PAUP* 4.0b2 (Swofford, 1999). All parsimony analyses were conducted using rigorous heuristic searches, i.e., 20 random addition sequences (all analyses found a single island sensu Maddison, 1991) and TBR swapping; gaps were treated as missing data. Multiple most parsimonious (MP) trees were combined as strict consensus trees.

In addition to standard measures of fit of characters to the resultant trees (consistency index, retention index), the strength of support for individual branches was estimated using bootstrap values (Felsenstein, 1985) and decay or Bremer indices (Bremer, 1988; Donoghue et al., 1992). For the nr ITS matrix, bootstrap (BS) values reported are from 200 full heuristic replicates with 20 random addition sequences and TBR branch swapping. The cp data provide poor resolution in some distal portions of the phylogeny such that most bootstrap replicates generated enough MP trees to swamp computer memory; it was thus not possible to use rigorous branch swapping methods. Instead, BS values are from 1000 replications with 500 random addition sequences each and no branch swapping. For both data sets, decay values for each branch were de-

Table 1.   Characteristics of the nuclear ribosomal ITS region (59 taxa) and chloroplast *trn*L-*trn*F (52 taxa) in Justicieae (outgroup taxa not included in these calculations). 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 *its*1 and *its*2, plus the 5.8s gene. [2] Includes the 3' *trn*F exon and 40 bp of the *trn*F gene that flanks the *trn*L-*trn*F spacer.

| | *its*1 | *its*2 | nr ITS region | *trn*L3' intron | Spacer | Intron + Spacer |
|---|---|---|---|---|---|---|
| Raw length | 193–278 | 211–234 | 410–512 | 347–521 | 224–332 | 710–824 |
| Aligned length | 356 | 288 | 644 (860[1]) | 629 | 473 | 1103 (1191[2]) |
| Variable sites (proportion) | 209 (0.59) | 176 (0.61) | 421 (0.49)[1] | 165 (0.26) | 160 (0.34) | 347 (0.31)[2] |
| Parsimony informative sites | 175 (0.49) | 114 (0.40) | 308 (0.36)[1] | 73 (0.12) | 78 (0.16) | 167 (0.15)[2] |
| Pairwise distances (range, %) | 0.8–31.5% | 0.5–33.2% | 0.5–23.8% | 0–9.6% | 0–15.9% | 0.4–10.9%[2] |
| GC content (range) | 0.66–0.77 | 0.66–0.77 | 0.67–0.76 | 0.33–0.38 | 0.36–0.42 | 0.35–0.38[2] |
| Scored indels, number | 12 | 2 | 14 | 7 | 10 | 17 |

termined 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. The difference between the length of these trees and the globally shortest trees is the decay index (DI) for the branch in question.

We obtained sequences for only one of the two loci for 13 taxa (see Appendix 1). Four of these (*Justicia brandegeana*, *J. spicigera*, *J. comata*, *Anisacanthus puberulus*) were sequenced for nr ITS but not the cp locus because sequences obtained earlier permitted us to judge that the more slowly evolving cp locus would be essentially invariant in these compared to close relatives (see McDade et al., 2000). DNA of the other taxa could not be amplified for the missing locus or more than ¼ of the sequence was missing even after attempts to sequence using all available primers. These taxa were included in the analysis of the locus for which complete (or nearly complete) data were available but were pruned from the data sets before combining. The data sets thus pruned to include complete sequences for the same set of 55 taxa (49 Justicieae + 6 outgroups) were combined into a single NEXUS file using the file editing capabilities of PAUP*. The nr ITS and cp *trn*L-*trn*F data sets were tested for congruence using Farris et al.'s (1995) Incongruence Length Difference test (implemented in PAUP* as the partition homogeneity test). Phylogenetic analyses of the combined data sets were conducted as described above; bootstrap values (200 replicates with 5 random addition sequences each) and decay indices (as previously described) were generated for each branch.

Alternative phylogenetic hypotheses were evaluated by using MacClade to prepare trees that reflect the relationships of interest. These were load-

ed into PAUP* as constraint trees, and the program 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 transitions) involved in accepting the alternative hypothesis.

To compare patterns of molecular evolution between loci and among lineages, matrices of pairwise HKY85 distances for both loci and for the combined sequences were output from PAUP* and moved into JMP (Sall & Lehman, 1996) for analysis. The relationship between pairwise distances for the two loci was examined using correlation. Rates of evolution were compared between selected sister lineages using a modified relative rates test (Sarich & Wilson, 1973). Distances between members of two sister lineages and their closest relative were tabulated, and *t*-tests were used to compare the means of these pairwise distances.

## RESULTS

### MOLECULAR EVOLUTION

Within the nr ITS region, *its*1 and *its*2 are roughly similar in variability except that *its*1 has more informative indels than *its*2 (Table 1). The cp *trn*L-*trn*F spacer is considerably more variable than the *trn*L3' intron, and the spacer is likewise more prone to indels although this difference is not marked. Whether considered in terms of overall variable sites or parsimony informative sites, the nr ITS region is twice as variable among these taxa as the cp *trn*L-*trn*F region (Table 1). Similarly, maximum pairwise distances are more than twice as great for the nr ITS sequences as for the *trn*L-*trn*F data. Across all taxa, pairwise distances for the two loci are positively correlated ($r = 0.669$, $N = 1176$, $P < 0.0001$), suggesting that although the two loci

are evolving at different rates, those rates are fairly consistent across Justicieae. Variation in rates of evolution among lineages will be discussed below in the context of phylogenetic relationships.

PHYLOGENETIC RELATIONSHIPS

Results of the partition homogeneity test indicate that the two data sets are not incongruent ($P = 0.34$). Further, except as regards taxa for which sequence data from only one locus were available, the topology obtained from the combined data set (Fig. 1) differs from the trees obtained from the separate data sets (not shown) only in degree of resolution or in weakly supported portions. For example, the nr ITS data do not resolve *Ptyssiglottis* T. Anderson as part of Isoglossinae, but do not support any other placement of this taxon. Similarly, the cp *trnL-trnF* data do not resolve relationships among most species of Old World *Justicia*, whereas the nr ITS data provided a fully (but weakly) supported hypothesis of relationships among these taxa. As expected, given congruence of the data sets and increased number of characters in the combined data set [308 and 218 parsimony informative characters in the nr ITS and cp *trnL-trnF* data sets, respectively, for a total of 526 in the combined data set (note that these tallies of parsimony informative characters are from the data sets pruned to include taxa for which both sequences were available)], branch support increases markedly in the combined topology. For all of these reasons, discussion of relationships is based on the outcome of the combined analysis.

Figure 1 presents the strict consensus of most parsimonious (MP) trees produced by analysis of the combined data sets. Note that only major lineages within Justicieae are labeled to emphasize higher level patterns of relationship; Figures 2 and 3 provide detail on relationships within lineages. In our discussion of relationships, when lineages identified here largely conform to previously recognized taxa, we use names of these established taxa; when lineages have not been previously recognized or named, we use informal names.

The combined analysis provides strong support for monophyly of Justicieae (Fig. 1, BS = 100, DI = 17). Within Justicieae, five lineages are moderately, e.g., the *Pseuderanthemum* lineage, BS = 75, DI = 2, to very strongly, e.g., the *Tetramerium* lineage, BS = 100, DI = 28, supported as monophyletic. The *Pseuderanthemum* lineage is sister to all other Justicieae, which are strongly supported as monophyletic (BS = 100, DI = 12). Isoglossinae are moderately well supported as monophyletic (BS = 82, DI = 3), and as sister to all Justicieae excluding the *Pseuderanthemum* lineage (BS = 89, DI = 3). The *Tetramerium* lineage is very strongly supported as monophyletic; this group is sister to a monophyletic lineage that includes Diclipterinae and *Justicia* and close relatives. These taxa, i.e., all Justicieae above the *Tetramerium* lineage exclusive of Diclipterinae and inclusive of both Old and New World plants, will be referred to subsequently as "justicioids." The labels New World "justicioids" and Old World "justicioids" will be used to refer to geographically delimited assemblages. The "justicioids" and Diclipterinae lineage is strongly supported as monophyletic (BS = 97, CI = 6). There is strong support for monophyly of the New World "justicioid" lineage (BS = 100, DI = 12) and of Diclipterinae (BS = 100, DI = 9), and for the sister-group relationship of these lineages (BS = 94, DI = 7). However, Old World "justicioids" are placed as a paraphyletic assemblage below New World "justicioids" + Diclipterinae.

Figures 2 and 3 are strict consensus trees showing placement of all included taxa, and bootstrap and decay support for all branches. Taxa for which sequence data for only one locus was available have been added to Figures 2 and 3 using stylistic conventions, i.e., branches are angled and taxon labels and support values are indicated in smaller type, to signal that the result is based on partial data. One randomly chosen MP tree is presented as Figure 4 to illustrate branch lengths.

*Pseuderanthemum lineage.* There is only modest support for monophyly of this lineage from the combined analysis (BS = 75, DI = 2). Nr ITS data indicate that *Spathacanthus* Baill. belongs here, and that the two included species are each other's closest relatives with strong support (Fig. 2; BS = 89, DI = 4). Our data do not resolve relationships

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Figure 1. Major patterns of relationships among Justicieae from parsimony analysis of combined nr ITS and cp *trnL-trnF* sequence data. Strict consensus of 24 most parsimonious trees (length = 2455, CI = 0.557, RI = 0.690). Bootstrap and decay indices are presented for major lineages only (see Figs. 2 and 3 for details of relationships within lineages and support for these). Note that all labeled lineages are monophyletic (indicated by solid vertical lines) with the exception of Old World "justicioids," which are a grade (indicated by dashed vertical line), as discussed in the text.
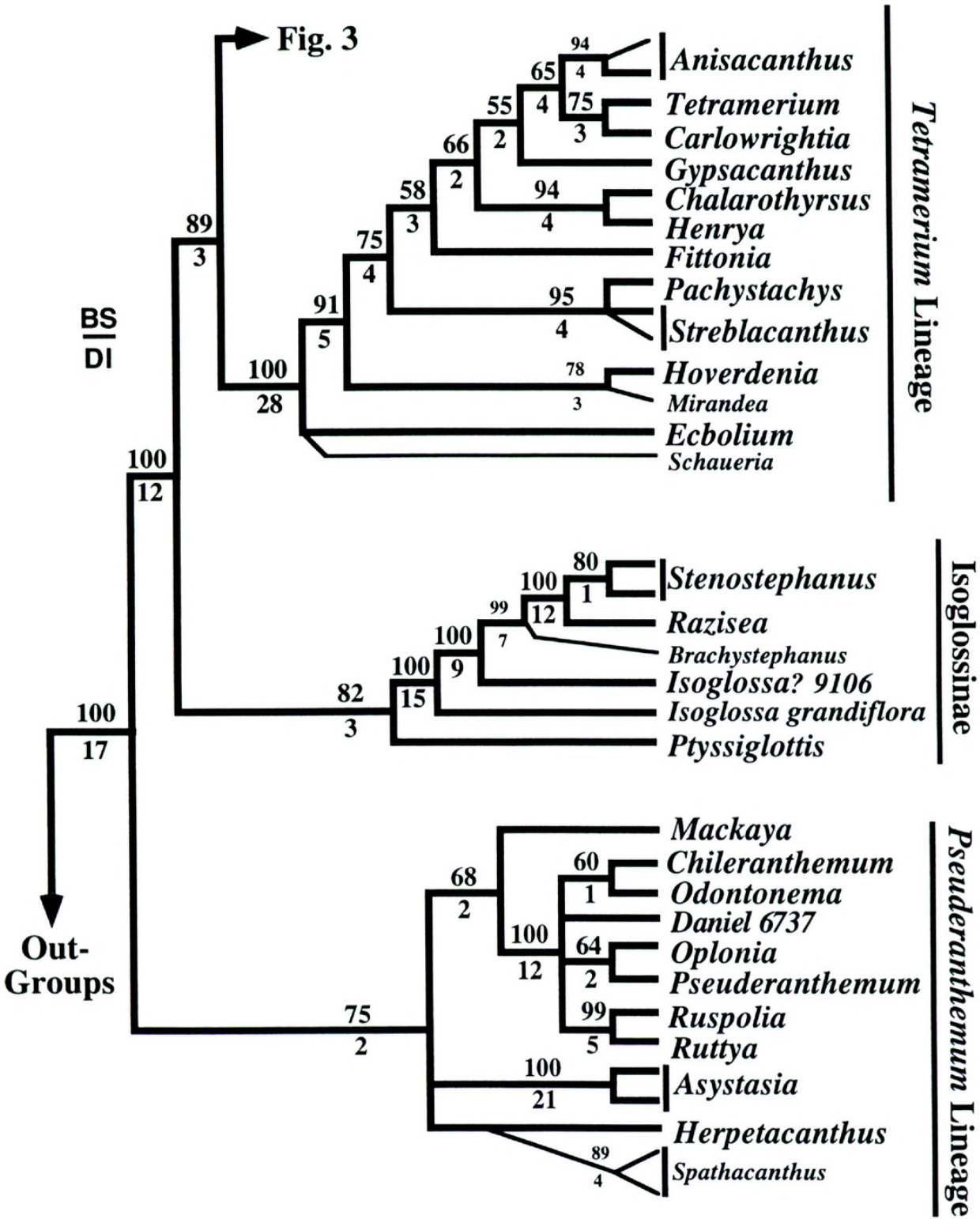
BS
—
DI
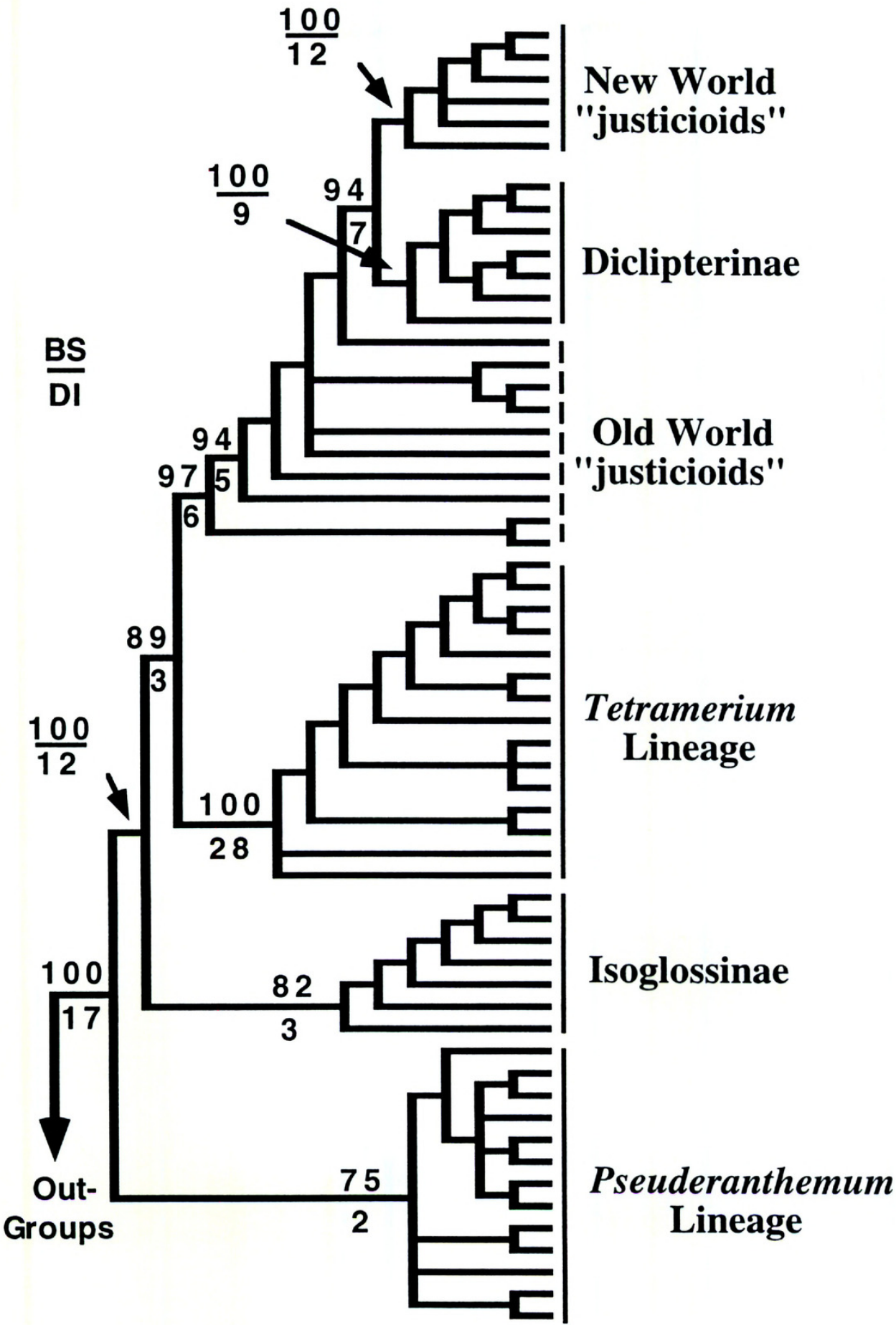
Figure 2. Relationships among members of the *Pseuderanthemum* lineage, Isoglossinae, and the *Tetramerium* lineage. Vertical lines link congeneric species when these are monophyletic. Taxa with bold, large typeface labels on thick branches are in the combined analysis (nr ITS + cp *trn*L-*trn*F); taxa for which data for only the nr ITS was available are added on thinner, angled branches with smaller typeface labels. Data for both loci were obtained for *Anisacanthus thurberi* and *Streblacanthus cordatus*, whereas only nr ITS data were obtained for a second member of each genus (*A. puberulus* and *S. roseus*; see Appendix 1). Bold, large typeface bootstrap and decay values are from the combined analysis; those in smaller typeface are from the analysis of the nr ITS data alone.
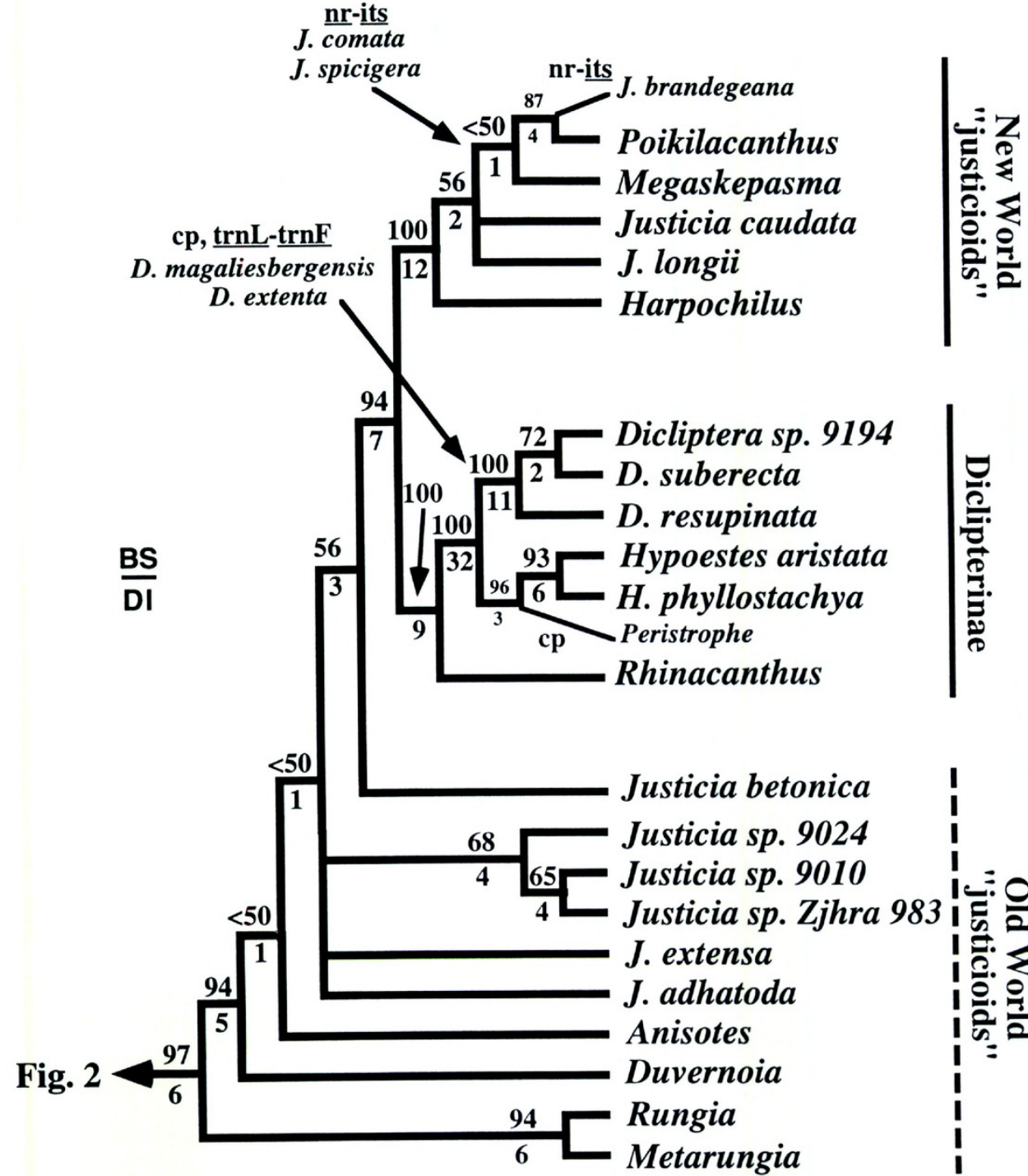
Figure 3.   Relationships among Old World "justicioids," Diclipterinae, and the New World "justicioid" lineage. Taxa with bold, large typeface labels on thick branches are in the combined analysis (i.e., nr ITS + cp *trn*L-*trn*F); taxa for which data for only one locus was available are added on thinner, angled branches or to the upper left. Bold, large typeface bootstrap and decay values are from the combined analysis; those in smaller typeface are from separate analyses of either the nr ITS or cp *trn*L-*trn*F data set. The positions of *Justicia brandegeana* (nr ITS) and *Peristrophe* (cp *trn*L-*trn*F) are resolved and strongly supported, whereas *J. comata* and *J. spicigera* (nr ITS), *Dicliptera extenta* and *D. magaliesbergensis* (cp *trn*L-*trn*F) are placed in polytomies with other New World *Justicia* and with *Dicliptera*, respectively.

among basal groups in the lineage [i.e., *Spathacanthus* (nr ITS only), *Herpetacanthus* Nees, *Asystasia* Blume]. The two representatives of *Asystasia* are, however, strongly supported as monophyletic; these species also share two unique indels, one in each genic region. Further, there is considerable molecular divergence between the two *Asystasia* species, and between *Asystasia* and other members of the
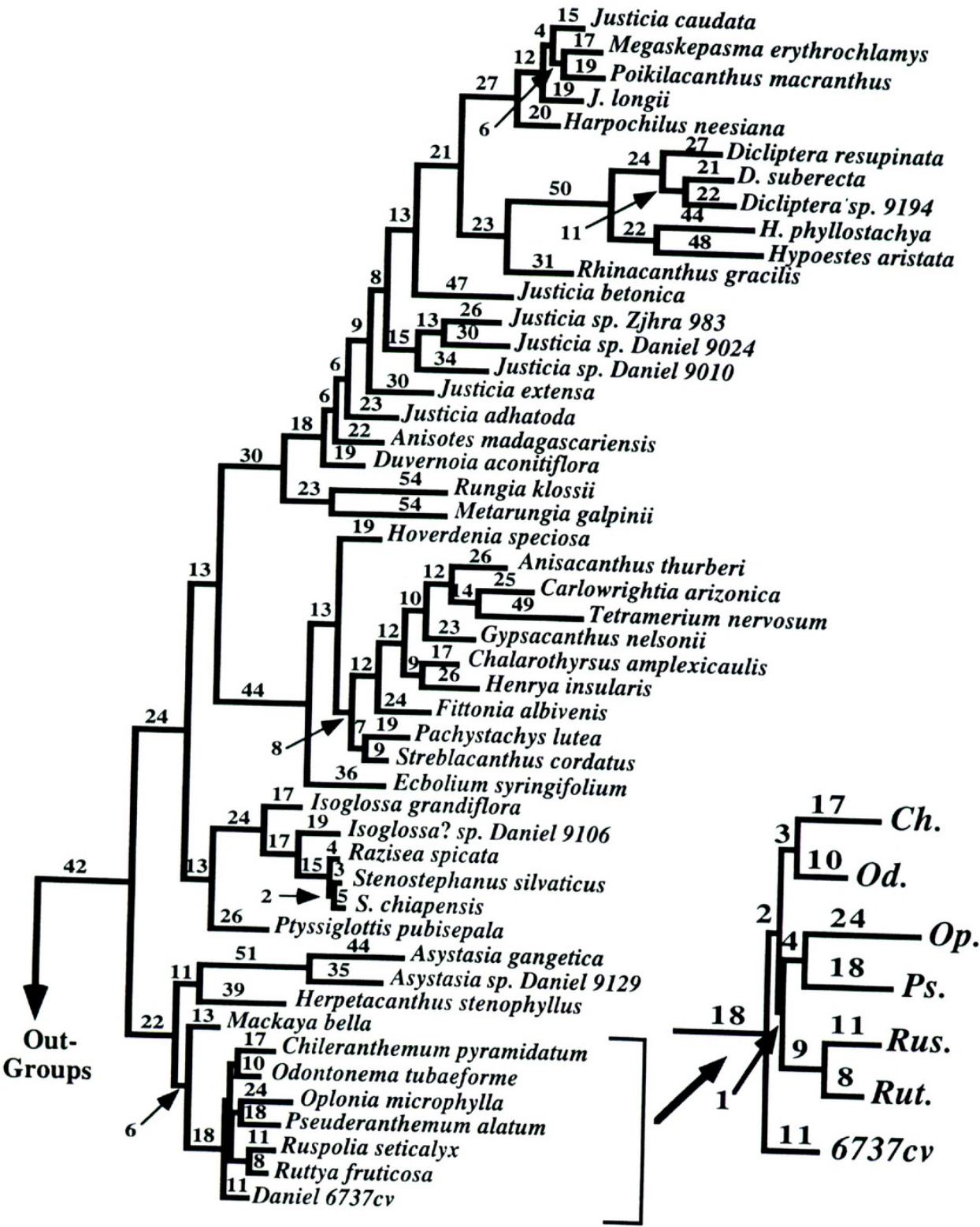
Figure 4. One (randomly chosen) of the most parsimonious trees from analysis of the nr ITS and cp *trn*L-*trn*F combined sequence data for all taxa for which data for both loci were available. Branch lengths are proportional to number of changes using ACCTRAN optimization; numbers on branches report branch length.

*Pseuderanthemum* lineage (note branch lengths in Fig. 4). Above this unresolved group of basal genera, there is weak support for *Mackaya* Harv. as sister to all others (BS = 68, DI = 2), and strong support for monophyly of the lineage above *Mack-*

*aya* (BS = 100, DI = 12). This last group includes one Malagasy species (*Daniel 6737cv*) that corresponds to specimens at P that were annotated as "*Justicia petiti* Benoist." It does not, however, appear that Benoist ever formally described this tax-

on, and it remains either unnamed or described with another name. There is little molecular divergence within the lineage above *Mackaya* (note short branch lengths, Fig. 4), and only the sister taxa *Ruspolia* Lindau + *Ruttya* Harv. are resolved with strong support (BS = 99, DI = 5).

*Isoglossinae.* A lineage that largely conforms to Lindau's (1895) Isoglossinae is identified by the combined analysis as monophyletic with modest support (BS = 82, DI = 3). The group of genera above *Ptyssiglottis* is extremely strongly supported as monophyletic (BS = 100, DI = 15). The Old World genus *Isoglossa* Oerst. may not be monophyletic: a putative species of *Isoglossa* (i.e., *Daniel 9106*) is more closely related to *Stenostephanus* Nees, *Razisea* Oerst., and *Brachystephanus* Nees (BS = 100, DI = 9) than to *I. grandiflora*. The nr ITS data place Old World *Brachystephanus* with New World *Razisea* + *Stenostephanus*, with strong support (BS = 99, DI = 7; note that these support values are from the nr ITS analysis alone). The two species of *Stenostephanus* are placed together, but with only modest support (BS = 80, DI = 1). There is, however, strong support for monophyly of *Razisea* + *Stenostephanus* (BS = 100, DI = 12). Short branch lengths among *Razisea* and *Stenostephanus* (Fig. 4) indicate that there is little molecular divergence among these three species.

*Tetramerium lineage.* The *Tetramerium* lineage is extremely strongly supported as monophyletic (BS = 100, DI = 28). Nr ITS data indicate that *Schaueria* Nees is part of this lineage but do not resolve its relationships. Further, the nr ITS data place the two sampled species of *Streblacanthus* Kuntze as sister taxa, and also place the two sampled species of *Anisacanthus* together (cp data not available for *S. roseus* and *A. puberulus*). There is strong support for monophyly of a lineage above *Ecbolium* Kurz (and *Schaueria*, nr ITS data only) (BS = 91, DI = 5), and moderate support for the lineage above *Hoverdenia* Nees (and *Mirandea* Rzed., nr ITS data only) (BS = 75, DI = 4). The combined data set provides strong support for sister-group relationships of *Streblacanthus* + *Pachystachys* Nees (BS = 95, DI = 5) and *Chalarothyrsus* Lindau + *Henrya* Nees ex Benth. (BS = 94, DI = 4), and nr ITS data provide moderate support for *Hoverdenia* + *Mirandea* (BS = 78, DI = 3; note that these values are from the nr ITS data alone). Although there is weak support for the internal nodes of the phylogeny distal to *Pachystachys* + *Streblacanthus*, monophyly of a lineage including *Anisacanthus*, *Tetramerium*, *Carlowrightia*, and *Gypsacanthus* E. J. Lott, V. Jaram. & Rzed. seems highly likely based on a shared deletion in the

spacer region of *trn*L-*trn*F that is ca. 150 bp in length.

*"Justicioids" and Diclipterinae.* There is strong support from the combined analysis (Fig. 3) for a clade comprised of much of Lindau's (1895) Diclipterinae plus all included "justicioids" (i.e., species of *Justicia* and the allied genera *Rungia* Nees, *Metarungia* Baden, *Duvernoia* E. Mey. ex Nees, *Anisotes* Nees from the Old World; *Harpochilus* Nees, *Megaskepasma* Lindau, and *Poikilacanthus* Lindau from the New World) (BS = 97, DI = 6). Within this lineage, the representatives of *Justicia* are not monophyletic. Instead, the Old World species are a paraphyletic assemblage that is basal to the Diclipterinae (as here circumscribed, see below) + the New World "justicioids" lineage. New World *Justicia* are monophyletic only if *Poikilacanthus* and *Megaskepasma* are included. Old World genera traditionally aligned with *Justicia* are placed as a series of lineages basal to Old World *Justicia*, with strong support for *Rungia* + *Metarungia* (BS = 94, DI = 6), for monophyly of all others above this pair (BS = 94, DI = 5), and then weak support for precise placement of *Duvernoia*, *Anisotes*, and the Old World representatives of *Justicia*.

There is strong support for monophyly of the Diclipterinae + New World "justicioids" lineage (BS = 94, DI = 7), and very strong support for monophyly of each of these lineages (Fig. 3). The lineage referred to here as Diclipterinae largely conforms to the core of Lindau's taxon of that name. *Rhinacanthus* Nees (not part of Lindau's Diclipterinae) is strongly supported as a basal member of the lineage, with very strong support for monophyly of all included taxa above *Rhinacanthus* (BS = 100, DI = 32). *Hypoestes* is monophyletic with strong support (BS = 93, DI = 6), and the cp *trn*L-*trn*F data place *Peristrophe* as sister to *Hypoestes*. The genus *Dicliptera* is monophyletic, including both Old and New World members. Especially above *Rhinacanthus*, branch lengths are notably longer in Diclipterinae than in adjacent groups (Fig. 4). Using *Justicia betonica* as the outgroup, a relative rates test indicates that sequence divergence in Diclipterinae is higher than in its sister group (the New World "justicioid" lineage). This is true for the loci considered separately (nr ITS: t = 3.229, 13 df, P = 0.0066; cp *trn*L-*trn*F: t = 3.89, 12 df, P = 0.0021) as well as for the combined data (t = 3.838, 9 df, P = 0.004).

New World "justicioids" (i.e., representatives of *Justicia* plus *Harpochilus*, *Megaskepasma*, and *Poikilacanthus*) are together monophyletic, with strong support (Fig. 3, BS = 100, DI = 12). Nr ITS sequence data place *J. brandegeana* as sister to *Po*-

*ikilacanthus* with strong support (BS = 87, DI = 4, nr ITS data alone), and place *J. comata* and *J. spicigera* as part of the clade that includes all other New World *Justicia*, but without resolution. Figure 4 indicates that branch lengths are quite short among members of the New World "justicioid" lineage, and a relative rates test shows lower levels of sequence divergence in this clade compared to its sister group, Diclipterinae.

## DISCUSSION

Justicieae are strongly supported as monophyletic in our analysis, and this has been confirmed in other analyses with richer samples of other Acanthaceae (McDade & Moody, 1999; McDade et al., 2000). This result is supported by nucleotide substitutions as well as by indels: all Justicieae included here share three indels in the cp locus. Unusual tricolporate hexapseudocolpate pollen grains (see figs. 7–10 in Daniel, 1998a) occur in all lineages of Justicieae as here delimited (although shifts to other types of grains are synapomorphies for some clades as discussed below). Pollen grains of this type are not known among Acanthaceae outside of Justicieae and, to our knowledge, are not known in other angiosperms. We here propose this character as a synapomorphy for Justicieae. Bremekamp (1965) proposed that this pollen type characterizes plants of his subtribe Odontoneminae; it is thus not surprising that his taxon is not monophyletic but instead assembles a heterogeneous group of Justicieae that lack more derived pollen types.

Lack of hygroscopic trichomes on the seeds characterizes Justicieae but may not be a synapomorphy. Seeds of plants of Ruellieae s.l., the sister group of Justicieae (see McDade et al., 2000), have hygroscopic trichomes (exceptions are genera previously included in Louteridieae and Trichanthereae, which have apparently lost these structures). Seeds of plants of Barlerieae + Whitfieldieae (these together comprise the sister group of Justicieae + Ruellieae s.l.; see Manktelow et al., in press) also have hygroscopic trichomes, again with a few exceptions in which they have apparently been lost secondarily. However, there is evidence that the hygroscopic trichomes differ between Ruellieae s.l. and Barlerieae + Whitfieldieae such that they are not likely homologous (Grubert, 1974; Scotland et al., 1995; Manktelow, 1996). If this is the case, then the common ancestor of these lineages would have lacked hygroscopic trichomes. Regardless of the phylogenetic status of the character, lack of hygroscopic trichomes distinguishes Justicieae from

plants of the other two lineages, with just a few exceptions. Acanthoideae also lack hygroscopic trichomes on the seeds, but these plants lack also the synapomorphies that link the other three lineages of Acanthaceae s. str., i.e., Ruellieae s.l., Barlerieae, Justicieae (e.g., cystoliths, articulated stems, colporate or porate pollen) and are thus unlikely to be confused with them.

*Pseuderanthemum lineage.* The position of these taxa as basal within Justicieae is clear from our data, but there is only modest support for their monophyly. Further, we know of no morphological synapomorphies for the entire lineage. Compared to other Justicieae, these plants have an androecium of four staminal elements (all four may be fertile or two may be reduced to staminodes). However, this is no doubt plesiomorphic for Justicieae: it is hypothesized to be a synapomorphy for all Lamiales s.l. (i.e., sensu Olmstead et al., 1993), and the other major lineages of Acanthaceae include taxa with this trait. Additional data will be necessary to test whether the lineage is indeed monophyletic or instead a series of lineages at the base of Justicieae. Most aspects of relationships within the *Pseuderanthemum* lineage are not resolved with confidence by our data. There is little divergence among most of these taxa for the loci examined here, and it will be necessary to work with morphological characters or more rapidly evolving loci to make progress in unraveling these phylogenetic relationships.

There is some corroborating evidence for those aspects of relationships within the *Pseuderanthemum* lineage that do emerge from our analysis. Whereas plants placed in unresolved fashion at the base of the lineage have four fertile stamens, two of these are reduced to staminodes in plants of the *Mackaya–Ruttya* clade. Further, although chromosome numbers have not been determined for *Chileranthemum* Oerst. or for the unidentified Malagasy taxon represented by *Daniel 6737cv*, all other genera in the *Mackaya–Ruttya* clade appear to have a base chromosome number of $x = 21$ (Daniel & Chuang, 1989; Daniel et al., 1990; Daniel & Chuang, 1993, 1998). The only chromosome number known for *Mackaya* (Old World, 2 species), $n = 42$, is from a single count by Daniel and Chuang (1989); $n = 42$ has not been recorded for any other genus in the lineage. A chromosome complement of $n = 21$ has been infrequently reported in genera representing various other lineages within the family, but does not seem to characterize other large, suprageneric groups. Below the *Mackaya–Ruttya* clade in the *Pseuderanthemum* lineage, chromosome counts are not available for *Herpetacanthus* (New World, ca. 10 species) or *Spathacanthus* (Neo-

tropical, 3 species), and $x = 21$ may thus be a synapomorphy for a more inclusive lineage. Interestingly, *Asystasia* (Old World, ca. 50 species) has $x = 13$ and includes species with polyploid derivatives of that number (Daniel, 2000). Several authors have suggested that $x = 7$ is symplesiomorphic for Acanthaceae (Grant, 1955; Raven, 1975; Piovano & Bernardello, 1991; Daniel & Chuang, 1993). If so, $x = 13$ has evolved via both polyploidy and dysploidy, and $x = 21$ may represent a hexaploid derivative of this base number.

There is very strong molecular support for the *Chileranthemum–Ruttya* clade but, within that clade, the only strongly supported relationship is that of *Ruspolia* and *Ruttya* as sister taxa. These two genera differ from others in the *Chileranthemum–Ruttya* clade in having monothecous stamens. *Ruspolia* (Africa and Madagascar, 4 species) and *Ruttya* (Africa, 3 species) are clearly closely related based on both molecular and morphological evidence, and a natural intergeneric hybrid, ×*Ruttyruspolia* A. Meeuse & de Wet, is known between them.

Heterostyly has been reported or observed by TFD or LAM in all genera of the *Chileranthemum–Ruttya* clade except *Ruttya* (based on the limited material available, it is not possible to determine whether *Daniel 6737cv* was collected from a heterostylous population). Given that this trait has only recently been observed in *Ruspolia* (LAM, pers. obs.), its absence in *Ruttya* may reflect lack of study rather than absence of the character. Heterostyly is otherwise unknown among Acanthaceae [the stylar polymorphism reported by Long (1971) for *Ruellia caroliniensis* (Walter) Steud. is related to corolla size but not to anther position]. As noted by Daniel (1995a), distinctions among *Chileranthemum* (New World, 3 species), *Odontonema* Nees (New World, ca. 30 species), *Oplonia* Raf. (American tropics and Madagascar, 14 species), and *Pseuderanthemum* Radlk. (pantropical, ca. 60 species) pertain primarily to differences in form of the corolla, which likely represent adaptations for different pollinators. These traits are well known to vary at low taxonomic level in Acanthaceae (e.g., Ezcurra, 1993, for *Ruellia*; Graham, 1988, for *Justicia*). We know of no diagnostic morphological features for these genera, and the monophyly of each should be tested.

Based on both molecular and morphological data, it seems clear that *Daniel 6737cv* should not be placed in *Justicia*; like other plants in the *Pseuderanthemum* lineage, it has an androecium of four staminal elements (two stamens plus two staminodes in this case, in contrast to *Justicia*, which lacks staminodes), and tricolporate hexapseudocolpate pollen (which is rare in *Justicia*). Like many Malagasy plants, its taxonomic placement requires additional study; our results do not indicate a clear generic assignment for the species.

The *Pseuderanthemum* lineage is a taxonomically heterogeneous group, including representatives of Lindau's (1895) tribes Asystasieae (*Asystasia*, *Spathacanthus*), Graptophylleae (*Ruspolia*), Isoglosseae (*Herpetacanthus*), Odontonemeae [*Chileranthemum*, *Mackaya*, *Odontonema*, *Ruttya*, and *Oplonia* (as *Anthacanthus* Nees)], and Pseuderanthemeae (*Pseuderanthemum*). To complete our understanding of this lineage and to test its monophyletic status, it will be important to include representatives of all Justicieae that have four fertile stamens or two stamens and two staminodes. As will be clear from his classification of the genera included here, Lindau (1895) distributed plants with four staminal elements in a number of tribes including Asystasieae (e.g., *Asystasiella* Lindau, *Chameranthemum* Nees, and *Thomandersia* Baill. in addition to those already included here), Graptophylleae (*Graptophyllum* Nees), Isoglosseae (*Podorungia* Baill., *Chlamydacanthus* Lindau, *Forcipella* Baill.), Odontonemeae (*Ballochia* Balf. f., *Phialacanthus* Benth., *Filetia* Miq.), and Pseuderanthemeae (*Codonacanthus* Nees). Many of these genera have not been studied in the century since Lindau's (1895) classification was proposed. More recently described genera with four staminal elements include *Pranceacanthus* Wassh. and *Pulchranthus* V. M. Baum, Reveal & Nowicke. Based on our results we predict that plants in most of these genera will be placed either as part of a monophyletic *Pseuderanthemum* lineage or as a series of basal lineages within Justicieae. It would also be useful to test monophyly of some of the larger genera in this lineage, especially those that are pantropical in distribution (*Pseuderanthemum*) or that have disjunct ranges (*Oplonia*, with 5 Malagasy and 9 New World species). Given the uncertainties noted above regarding exact taxonomic composition, we estimate that the *Pseuderanthemum* lineage comprises about 200 species.

As noted above, whether or not the *Pseuderanthemum* lineage is monophyletic, there is very strong support from our analysis for monophyly of all other Justicieae. These plants have an androecium of two stamens; that is, with only two exceptions of which we are aware, they have lost even staminodial remnants of the other pair. Remarkably, plants of *Chalarothyrsus* (here placed as a member of the *Tetramerium* lineage, see below) have four dithecous stamens. Two species of *Ptyssiglottis*

(here placed as the basal member of Isoglossinae, see below) are reported to have small or remnant staminodes (Hansen, 1992).

*Isoglossinae.* Lindau's (1895) key to tribes and subtribes distinguishes Isoglossinae based on the presence of two stamens with mono- or dithecous anthers and "Gürtelpollen" (i.e., girdled pollen). However, in describing the subtribe and in assigning genera to it, he included also some plants that lack this pollen type and have four stamens (e.g., *Herpetacanthus, Populina* Baill.). As discussed below, our results suggest that Lindau was correct in recognizing a group marked by girdled pollen. He was also correct in assigning additional genera that lack this synapomorphy to Isoglossinae, but erred in at least some assignments, e.g., *Herpetacanthus* is a member of the *Pseuderanthemum* lineage.

In our analysis, *Ptyssiglottis* (southeastern Asia to Papuasia, 33 species) is placed in Isoglossinae with moderate support. These plants have dithecous anthers and a diversity of pollen types including the basic type for Justicieae (tricolporate hexapseudocolpate pollen), but not the girdled biporate pollen characteristic of "core Isoglossinae" (Hansen, 1992; see below). *Ptyssiglottis* was placed by Lindau (1895) in Pseuderanthemeae, probably on the basis of its pollen. As delimited by Hansen (1992) plants of *Ptyssiglottis* lack staminodia with the two exceptions noted above of small or remnant staminodia. We are, however, unable to point to clear, non-molecular synapomorphies linking this genus to other Isoglossinae. Hansen (1992) treated *Ptyssiglottis* as a member of Isoglossinae but did not describe synapomorphies supporting the relationship. It would be well to test the present hypothesis that *Ptyssiglottis* is part of Isoglossinae by additional data (DNA sequence or otherwise). If this is the case, then the genus can be viewed as transitional between more typical Justicieae and "core Isoglossinae," which are marked by distinctive pollen morphological synapomorphies.

"Core" Isoglossinae (i.e., *Isoglossa* through *Stenostephanus*) are extremely strongly supported as monophyletic in our analysis. In addition to sequence data, this clade is marked by three unique and unreversed indels (two in the nr ITS locus). These plants also appear to share Lindau's "Gürtelpollen," which is typically biporate with pores that are surrounded by a more or less circular region. The two circular regions are separated from one another by a peripheral band (continuous or interrupted) of varying width. This type of pollen is described and figured by Daniel (1999) for *Stenostephanus* and *Razisea*. Similar pollen has been described in *Brachystephanus* (Figueiredo & Keith-

Lucas, 1996) and *Isoglossa* (Muller et al., 1989; Raj, 1961, as *Rhytiglossa* Nees ex Lindl.), although at least the former genus shows more diversity in features such as aperture number. The identity of *Daniel 9106* from Madagascar has yet to be determined, but preliminary examination of its pollen reveals biporate pollen like that of other "core" Isoglossinae, and its macromorphological characteristics suggest its placement in *Isoglossa*. If it indeed represents a species of this genus, then the two species of *Isoglossa* included in our analysis do not form a monophyletic group. Instead, *Daniel 9106* is more closely related to *Stenostephanus, Razisea*, and *Brachystephanus* than to *I. grandiflora*. Interestingly, in addition to sequence support for this relationship, *Daniel 9106* also shares two indels in the *trnL-trnF* region with *Stenostephanus + Razisea* that *I. grandiflora* lacks. The nr ITS data support monophyly of *Brachystephanus* (tropical Africa and Madagascar, 13 species) and Neotropical *Stenostephanus* (ca. 75 species) and *Razisea* (4 species). These three genera share monothecous anthers as a morphological synapomorphy. The New World members of this sublineage have been divided into a series of small genera reflecting remarkable floral diversity (e.g., *Cylindrosolenium* Lindau, *Kalbreyeracanthus* Wassh., *Kalbreyeriella* Lindau). Wood (1988) treated most of these as congeneric with *Habracanthus* Nees, and Daniel (1995b, 1999) further combined *Habracanthus* with *Stenostephanus*. Nomenclatural changes are gradually being made to reflect this recasting of generic limits, e.g., Wasshausen (1999). Our results indicate that there is considerable merit to this approach and confirm that *Razisea* is part of this group as well (Daniel, 1999). However, investigation of relationships among the New World species using molecular data will require a locus evolving more quickly than those we have studied (note short branch lengths in Fig. 4).

Isoglossinae, as here defined, are particularly poorly known cytologically. Among genera studied by us, chromosome counts have been determined for only five species. It is noteworthy that the Neotropical genera *Stenostephanus* and *Razisea* appear to share a common chromosome number of $n = 18$ (Daniel, 1999). No chromosome numbers have been reported for the Old World genera *Brachystephanus* or *Ptyssiglottis*, and only a single count ($n = 17$) has been reported for the Old World genus *Isoglossa* (widespread in Old World tropics and subtropics, ca. 60 species) (Daniel & Chuang, 1998).

To delimit Isoglossinae clearly, it would be well to include representatives of other genera that lack the synapomorphies that mark "core" Isoglossinae.

Genera that were included in this subtribe by Lindau (1895, 1897) and that would appear to be part of "core" Isoglossinae as defined here include *Cylindrosolenium*, *Populina*, and *Oreacanthus* Benth. These, and other more recently described genera with two dithecous stamens and "Gürtelpollen" (e.g., *Conocalyx* Benoist, *Sphacanthus* Benoist), should be added. Acknowledging uncertainty about placement of some of these smaller genera, we estimate that Isoglossinae include about 200 species.

Monophyly of the larger genera of Isoglossinae should be tested, including *Ptyssiglottis*, *Isoglossa*, and *Stenostephanus*. Monophyly of *Brachystephanus* should be tested, and the usefulness of maintaining this genus separate from *Stenostephanus* also merits evaluation. Broad-scale studies of a number of taxa have demonstrated that morphologically similar genera in widely separated geographic regions are often congeneric (e.g., *Stenandrium* Nees–*Stenandriopsis* S. Moore, Vollesen, 1992; *Oplonia*–*Forsythiopsis* Baker, Stearn, 1971; *Mendoncia* Vell. ex Vand., *Afromendoncia* Gilg ex Lindau, and *Monachochlamys* Baker, Benoist, 1925).

Monophyly of all Justicieae above these first two lineages (i.e., *Pseuderanthemum* lineage and Isoglossinae, Fig. 1) is moderately supported (BS = 89, DI = 3), but we know of no non-molecular synapomorphies that mark this lineage.

*Tetramerium* lineage. Sequence data strongly support monophyly of this lineage; indeed, it is one of the most strongly supported clades in all of Justicieae. In addition to sequence data, all of the plants included in our analysis uniquely share five indels in the nr ITS region. The genera sampled here were placed by Lindau (1895) in diverse tribes: Asystasieae (*Chalarothyrsus*, part of *Henrya* [as *Solenoruellia* Baill.]), Graptophylleae (*Anisacanthus*, *Carlowrightia*, *Pachystachys*), Isoglosseae (*Fittonia*), and Odontonemeae [*Ecbolium*, *Hoverdenia*, *Schaueria*, *Streblacanthus*, *Tetramerium* (including *Henrya*)]. Despite Lindau's treatment and the fact that we can identify no clear non-molecular synapomorphies for the lineage, a group corresponding closely to that delimited here has been consistently identified based on monographic and cytological studies (Daniel, 1986, 1990; Daniel & Chuang, 1993; Daniel et al., 1984). A chromosome number of $n = 18$ seems to characterize the entire group, with additional diversity in some genera [e.g., *Ecbolium linneanum* has been reported to have $n = 14$ (one count), 18 (six counts), and 20 (one count); chromosome data are lacking for *Hoverdenia* and *Schaueria*]. Because $n = 18$ also occurs in Isoglossinae and in a few species of *Justicia*, it is difficult to determine whether it is a synapo-

morphy for the *Tetramerium* lineage or for a more inclusive group. These plants share a number of traits that are symplesiomorphic at this level within Justicieae: tricolporate hexapseudocolpate pollen (a synapomorphy for all Justicieae); an androecium of two bithecous stamens and no staminodes (a synapomorphy for Justicieae above the *Pseuderanthemum* lineage); and thecae that are parallel, inserted at more or less the same height on the filament, and unappendaged (these thecal characteristics are variously modified in many members of the "justicioids" and Diclipterinae lineage).

The placement of *Chalarothyrsus* within the *Tetramerium* lineage is surprising in that these plants have four bithecous stamens, a trait known to occur among Justicieae only in the *Pseuderanthemum* lineage, as here defined. However, *Chalarothyrsus* has a chromosome complement of $n = 18$ (contra $n = 21$, which characterizes the *Pseuderanthemum* lineage, see above). We can only conclude that there was a reversal to four functional stamens in the evolutionary history of plants of this unispecific genus from western Mexico. Given that occasional flowers with atypical androecia have been observed in a number of species of Acanthaceae (TFD & LAM, pers. obs.), such a reversal does not seem especially improbable. Further, trees placing *Chalarothyrsus* with the *Pseuderanthemum* lineage are 68 steps (3%) longer than the MP trees.

Within the *Tetramerium* lineage, most aspects of relationships are not strongly supported. Monophyly of all included genera above *Ecbolium* (Old World, 22 species) [+ *Schaueria* (New World, ca. 10 species), nr ITS data only] is strongly supported, and that of *Anisacanthus* through *Streblacanthus* is moderately well supported. We are not able to identify clear non-molecular synapomorphies for these lineages. There is moderate to strong support for sister relationships between *Hoverdenia* (Mexico, 1 species) + *Mirandea* (Mexico, 4 species) (nr ITS only), *Pachystachys* (West Indies and South America, 12 species) + *Streblacanthus* (Central and South America, 4 species), and *Chalarothyrsus* + *Henrya* (North and Central America, 2 species) but, again, non-molecular evidence is lacking. A clade comprised of *Anisacanthus* through *Henrya* (Fig. 2) is not especially strongly supported by sequence data (BS = 66, DI = 2), but is marked by a unique 22 bp deletion in the nr ITS region. Similarly, a clade composed of the New World genera *Anisacanthus* (ca. 20 species), *Tetramerium* (28 species), *Carlowrightia* (24 species), and *Gypsacanthus* (1 species) is only weakly supported by sequence data but, as noted above, these plants share a > 150bp deletion in the *trnL-trnF* spacer. This shared de-

letion argues strongly for their monophyly and also explains the weak support from sequence data: the cp region that is absent in these four genera is the most variable of the cp locus (i.e., the most likely source of nucleotide substitutions among close relatives). The paucity of morphological evidence for relationships in the *Tetramerium* lineage reflects Daniel and Chuang's (1993) statement regarding problematic generic delimitations among these plants. Generic boundaries are difficult at best and are mostly based on differences in floral morphology that reflect adaptation to pollinators (e.g., bees and flies in *Carlowrightia* and *Henrya*; hummingbirds in *Anisacanthus*). Among genera in this lineage, only *Henrya* and *Chalarothyrsus* have unambiguous apomorphies (i.e., fused bracteoles and pollen traits in the former, seeds fused to the capsule valves in the latter) that are not likely related to recent selection by pollinators.

Interestingly, this *Tetramerium* lineage is almost exclusively New World in geographic distribution. Mexico is especially rich in its members and several genera are endemic there [i.e., *Chalarothyrsus, Gypsacanthus, Hoverdenia, Mirandea, Aphanosperma* T. F. Daniel; (the last is not included in our sample but undoubtedly belongs here on the basis of macromorphological, palynological, and cytological similarities; Daniel, 1988, 1990)] and others have their greatest concentration of species in Mexico (e.g., *Anisacanthus, Carlowrightia, Henrya, Tetramerium*). *Ecbolium* is the only Old World member in the present sample, but its placement here is not surprising: Vollesen (as cited in Balkwill & Balkwill, 1998) and Daniel (1998b) have suggested that these plants are congeneric with the North American *Yeatesia* Small, a genus not included in our analysis but which clearly belongs in this lineage. The Old World genera *Megalochlamys* Lindau, *Angkalanthus* Balf. f., and *Calycacanthus* K. Schum. likely also belong here and should be included in future work (the same is true of New World *Yeatesia* and *Aphanosperma*, as noted above). Assuming that we are correct about placement of these genera in the *Tetramerium* lineage, the group includes about 150 species.

*"Justicioids" and Diclipterinae lineage.* Monophyly of the "justicioids" and Diclipterinae lineage is strongly supported in the combined analysis (recall that "justicioids" is used here to include *Justicia* and allied genera, both Old and New World in distribution). Among these plants, there is a marked tendency for increased complexity in anther morphology and ornamentation compared to other Justicieae. Thus, the thecae are usually inserted at different heights on the filament and not

perfectly parallel; basal appendages of various shapes and sizes occur on the thecae in many species. This lineage is also marked by evolution of a rugula: a channel-like structure on the internal surface of the upper lip formed by parallel ridges of corolla tissue. During anthesis, the style lies in this rugula, sometimes fitting so snugly that force is required to dislodge it. If we are correct in placing the evolution of the rugula here, then this structure is lost in Diclipterinae (as here delimited) above *Rhinacanthus* (see below). The genera not included in Diclipterinae are placed either as part of a non-monophyletic grade that includes Old World "justicioids" or as part of a clade, the New World "justicioids" lineage. As will be clear from the following discussion, our analysis confirms that generic delimitations are problematic among "justicioids," adding the additional complication that neither "justicioids" as a whole nor the genus *Justicia* are monophyletic.

*Old World "justicioids."* The taxa represented in our analysis are not monophyletic, nor do the species of Old World *Justicia* that we have included form a monophyletic lineage. However, it is interesting that all other Old World "justicioids" are basal to the six Old World species of *Justicia* that are included. The sister-group relationship between *Rungia* (Paleotropics, ca. 20 species) and *Metarungia* (Africa, 3 species) is strongly supported in our analysis. These plants share a placenta that rises elastically from the base of the capsule at maturity (this trait is also found in *Dicliptera*, see below). In plants of both genera, the bracts have a distinctive hyaline or colored border. There is strong support for monophyly of the lineage above *Rungia + Metarungia*, but we know of no non-molecular evidence for this relationship. Relationships among other Old World "justicioids" are not strongly supported by our analysis, although it is interesting that the three Malagasy *Justicia* are moderately well supported as monophyletic (BS = 68, DI = 4).

Conclusions regarding relationships among Old World "justicioids" are unwarranted based on our limited sample (10 of at least 300 species) and the inconclusive pattern of relationships among these. Further, all *Justicia* species from the Old World are monophyletic in trees that are only four steps (0.2%) longer than the MP trees. Similarly, constraining all Old World "justicioids" to monophyly requires only eight additional steps (0.3%) compared to the shortest trees. On the other hand, evidence from indels suggests that the present hypothesis is correct in placing some Old World "justicioids" closer to the Diclipterinae + New World "justicioid" lineage than to other Old World

"justicioids." The three species of Malagasy *Justicia* and *J. betonica* share a 3 bp indel in the cp locus with the Diclipterinae + New World "justicioids" lineage, and these plus *J. extensa* share a 7 bp indel, also in the cp locus, with the Diclipterinae + New World "justicioids" lineage.

Students of Acanthaceae have differing opinions regarding the validity of a number of genera that are clearly closely related to Old World *Justicia* (e.g., *Monechma* Hochst., *Adhatoda* Mill., *Aulojusticia* Lindau, Old World *Siphonoglossa* Oerst., *Ascotheca* Heine, *Trichocalyx* Balf.f., *Chlamydocardia* Lindau, and *Sarojusticia* Bremek.). Adding representatives of these groups will both expand our sample of Old World "justicioids" and test their validity as genera. The geographic range of Old World "justicioids" is extensive (Africa through west Asia to southeast Asia and Australia) and under-sampled here (we have included only African, Malagasy, and south Asian species). These plants present a series of intriguing biogeographic patterns that can be usefully addressed once we have a well resolved phylogeny for a denser and geographically broader sample. The phylogenetic status of *Justicia* is discussed further under "The *Justicia* Problem," below.

Strong support for monophyly of the Diclipterinae + New World "justicioid" lineage is perhaps the most surprising component of our results. To our knowledge, this relationship has not previously been suggested, and we cannot identify any clear non-molecular synapomorphies to corroborate the strong support from molecular sequence data. Certainly this hypothesis of relationships should be tested with additional data.

*Diclipterinae.* There is strong support from our analysis for a monophyletic Diclipterinae, including *Rhinacanthus*. This lineage conforms to the core of Lindau's (1895) Diclipterinae in which he placed *Peristrophe*, *Hypoestes*, and *Periestes* (= *Hypoestes*), in addition to *Dicliptera*. However, Lindau included also *Tetramerium* and *Rungia* here; we have shown that these genera have relationships elsewhere in Justicieae. Further, he placed *Rhinacanthus* in Odontoneminae. In fact, *Rhinacanthus* (Old World, ca. 20 species) shares a rugula with "justicioids" and lacks a number of the morphological synapomorphies that otherwise characterize Diclipterinae. Nonetheless, molecular data (including two unique and unreversed indels in the cp locus) strongly support *Rhinacanthus* as a member of this lineage, and cytological data corroborate this placement. Diclipterinae including *Rhinacanthus* seem to share a base chromosome complement of $x = 15$. Although chromosome numbers of $n = 15$ and $n = 30$ are

not uncommon elsewhere in the family, among Justicieae $n = 15$ is known only in a few species of *Justicia*. Among *Rhinacanthus*, *Peristrophe*, and *Hypoestes*, $n = 15$ and 30 are the most commonly reported numbers. Many Old World *Dicliptera* have $n = 13$, but $n = 15$ is known from both African and Malagasy species (Kaur, 1970; Daniel, unpublished data). We thus suggest that $n = 13$ represents dysploid evolution from $n = 15$. Interestingly, all New World species of *Dicliptera* for which counts have been obtained have $n = 40$ and thus appear to be ancient polyploids (Daniel, 2000; Daniel & Chuang, 1993).

Diclipterinae above *Rhinacanthus* are one of the most strongly supported lineages in our analysis; these plants are also well marked by morphological synapomorphies. Corollas of these plants lack the rugula that is otherwise characteristic of the "justicioids" and Diclipterinae lineage. *Hypoestes*, *Peristrophe*, and *Dicliptera* all share a specialized type of compound inflorescence (Balkwill & Getliffe Norris, 1988). Additionally, in these three genera the corolla is resupinate through 180 degrees of rotation. This trait has apparently been lost secondarily in a number of New World *Dicliptera*. Interestingly, in some species of New World *Dicliptera*, the corolla is resupinate through a full 360 degrees: in these plants, the corolla appears to be normally oriented, but this is not achieved in the normal way (Daniel, 1995c).

The two species of *Hypoestes* are strongly supported as each other's closest relative, and cp data place *Peristrophe* sister to these two, again with strong support (Fig. 3; these taxa also share an 11 bp indel in the cp locus). *Hypoestes* (Old World, ca. 150 species) differs from *Peristrophe* (Old World, ca. 25 species) and *Dicliptera* (Old and New World, ca. 80 species) by having monothecous anthers. We know of no non-molecular synapomorphies that mark the *Hypoestes* + *Peristrophe* clade.

Chloroplast data (including three indels, one ca. 50 bp long) place all five species of *Dicliptera* together, and the combined data set provides strong support for monophyly of the three species (including two from the New World and one from the Old World) for which we have sequence data for both genic regions. Species of *Dicliptera* have fruits with elastic dehiscence of the placentae; this distinguishes them from other Justicieae except *Rungia* + *Metarungia*, which also have this trait, as noted above. Our results agree with the conclusions of Balkwill and Getliffe Norris (1988) in indicating that elastic placentae evolved separately in these two groups; subtle differences in the trait are to be sought. In fact, fruits of a number of other genera

including *Tetramerium* and *Henrya* in Justicieae have placentae that separate from the fruit wall, but are not considered elastic (Daniel, 1986, 1990). These characters merit further comparative study across Acanthaceae to identify homologies and analogies.

Diclipterinae, with an estimated diversity of 300 species, have not suffered from the proliferation of small genera observed in other lineages of Justicieae. *Diclipera* and *Hypoestes* are large genera whose monophyly should be tested. Especially interesting is that the biogeographic range of *Diclipera* has a cytological correlate, as noted above; phylogenetic work within this genus is certainly warranted.

*New World "Justicioid" lineage.* Our results suggest the existence of a monophyletic group that includes all New World *Justicia* and members of related genera. In addition to sequence support for this relationship, all taxa share two indels in the cp locus. Generic limits among New World "justicioids" have been unsettled for some time. For example, among species included here, *J. brandegeana* was described originally in *Beloperone* and subsequently moved to its own genus, *Calliaspidia* Bremek., by Bremekamp (1948). *Justicia longii* was treated in *Siphonoglossa* until this group was studied by Hilsenbeck (1990). Our analysis indicates that submerging these genera in *Justicia* was warranted. In her infrageneric classification of *Justicia*, Graham (1988) included these and many other genera in *Justicia*. Daniel (1991) noted that *Poikilacanthus* (ca. 14 species) has but little to distinguish it from New World *Justicia* except pollen morphology, as was also noted by Raj (1961) and Bremekamp (1965). In fact, among species of New World *Justicia* there is already a remarkably rich diversity of pollen morphology (Daniel, 1998a). *Megaskepasma*, with a single Neotropical species, is a plant with spectacular magenta bracts, long white corollas, and pollen that is similar to at least one species of *Poikilacanthus* (Daniel, 1991, 1998a). Finally, *Harpochilus* (3 species) is a poorly known genus endemic to Brazil with highly specialized corollas. Morphological distinctions between this genus and *Justicia* have not been fully investigated.

We estimate that the New World "justicioid" lineage includes about 400 species, with much still remaining to be discovered about species-level diversity. In this context, our sample of eight species (including the three for which we have only nr ITS data) is extremely sparse. On the other hand, our sample is taxonomically diverse, as described above, such that we regard the hypothesis that the entire group is monophyletic as robust. However,

species representing other genera, both currently recognized as well as those already synonymized with *Justicia*, should be included (e.g., *Sebastiano-Schaueria* Nees, *Clistax* Mart., *Chaetochlamys* Lindau, *Chaetothylax* Nees, *Neohallia* Hemsl., *Tabascina* Baill.). On the other hand, as indicated by branch lengths in Figure 4, there is remarkably little molecular diversity among the sampled species. For both the nr ITS and *trnL-trnF* sequences, New World "justicioids" have lower rates of divergence than their sister group (Diclipterinae). These low levels of molecular divergence contrast with species diversity (the New World "justicioid" lineage is as species-rich as any other lineage of Justicieae), as well as with morphological and cytological disparity. Plants of this lineage range from prostrate herbs to trees, have corollas from a few mm to at least 8.5 cm in length, and have a startling range of diversity in pollen morphology (Daniel, 1998a). At least 11 different chromosome numbers, ranging from $n = 11$ to $n = 31$, have been reported for New World species of *Justicia* alone. It is remarkable that this explosion of morphological and cytological diversity is not reflected by molecular divergence, at least at these loci.

*The* Justicia *problem.* Our results indicate that *Justicia* is not monophyletic, that New World *Justicia* is monophyletic only if a number of other "justicioids" are included, and that Old World species of *Justicia* are unlikely to form a monophyletic group whether or not other "justicioids" are included. Clearly, a great deal remains to be learned about phylogenetic relationships of plants described in this genus. Even species-level diversity remains poorly documented among "justicioids": *Index Kewensis* (Davies, 1991, 1996) reports 81 new species described in *Justicia* alone between 1986 and 1995.

The results of our analysis point to a strong phylogenetic distinction between Old and New World "justicioids," despite the fact that they share many macromorphological characteristics, have similar chromosome number patterns, and the same wide range of pollen types. Constraining New + Old World "justicioids" to monophyly requires 32 additional steps (1.5%) compared to the shortest trees. There are no established benchmarks against which to evaluate an increase of 1.5% in tree length, but note that decay indices (Figs. 2, 3) indicate that only Diclipterinae above *Rhinacanthus* would be resolved in trees 30 steps longer than the MP trees. That is, trees in which all "justicioids" are monophyletic would lack resolution among essentially all other Justicieae.

In her infrageneric classification of *Justicia*

based on study of 295 species of the genus, Graham (1988) recognized 16 sections. Interestingly, Graham's work seems to have presaged our results in that none of her sections have species in both hemispheres (7 are restricted to the Old World, 9 to the New World). However, the characters used to distinguish the sections often seem extremely fine [e.g., the distinctions between the Old World sect. *Justicia* and the New World sect. *Drejerella* are based on corolla color (cream versus red) and size (less than 25 mm versus more than 35 mm)]. We have thus tended to view her work as a monumental step toward characterizing variation among species of *Justicia*, but still preliminary. Our sample of "justicioids" does not permit evaluation of Graham's (1988) sections, but she seems to have been correct in separating New and Old World members. Clearly, our own contribution can be viewed as preliminary as well in that we are still far from a full understanding of relationships among all "justicioids."

## Conclusions

The morphological diversity among Justicieae as well as the sheer size of the group in terms of number of species has made coming to terms with infratribal relationships quite challenging. In such diverse groups, the simplicity of molecular sequence data simplifies comparison. More importantly, placing comparative data in a phylogenetic context permits distinguishing shared derived characters that argue for relationships from shared primitive characters that do not.

It will be clear from the foregoing discussion that Lindau (1895) correctly diagnosed Diclipterinae and Isoglossinae (although he placed in both of these subtribes some genera that we demonstrate belong elsewhere) but otherwise shuffled things up rather thoroughly. Bremekamp (1965) was likewise correct in recognizing Isoglossinae (his Rhytiglossinae) and Justiciinae. The fact that he did not include Diclipterinae in the latter is mirrored by our inability to point to non-molecular synapomorphies that support the embedding of Diclipterinae in Bremekamp's Justiciinae (our "justicioids"). Bremekamp's Odontoneminae thus included Diclipterinae (except *Rhinacanthus*), the *Tetramerium* lineage, and the *Pseuderanthemum* lineage, a non-monophyletic group of plants whose morphological basis lies in retention of primitive characters. More recently, Balkwill and Getliffe Norris (1988) presented a classification for southern African Acanthaceae that improves upon Bremekamp's classification by removing Diclipterinae (again, as here defined except that *Rhinacanthus* is placed with their Justiciinae) from Odontoneminae. These authors also recognize Isoglossinae, Justiciinae (our Old World "justicioids" plus *Rhinacanthus*), and Odontoneminae. This latter group includes elements of our *Pseuderanthemum* and *Tetramerium* lineages. Finally, our results corroborate the findings of McDade and Moody (1999) and McDade et al. (2000) regarding clades within Justicieae, while expanding the sample of Justicieae considerably. In particular, these earlier analyses included no Old World "justicioids."

As discussed throughout, our results are limited in a number of ways. First, although much of our phylogenetic hypothesis is remarkably strongly supported, a few key aspects are not. Notably, establishing the phylogenetic status of the *Pseuderanthemum* lineage with confidence requires additional research effort. Second, in some lineages (notably most of the *Pseuderanthemum* lineage and New World "justicioid" lineage), the genic regions examined here do not provide sufficient variation to elucidate relationships with confidence. It will be necessary to add another, more rapidly evolving locus to our molecular tools in order to unravel relationships in these groups and at lower taxonomic levels than explored here. Third, sampling remains insufficiently dense to address all of the interesting phylogenetic problems in Justicieae, notably with regard to Old World "justicioids." Stating this shortcoming more positively, our results provide a framework to which additional taxa can be readily added to address specific questions regarding phylogenetic relationships. In addition to taxa whose characters permit predictions about their placement into this framework, a few present character combinations that defy predictions (e.g., *Leandriella* Benoist with biporate pollen similar to "core" Isoglossinae and four stamens characteristic of basal Justicieae). Fourth, in many cases, our knowledge of morphology, palynology, and cytology among Justicieae remains inadequate for the sort of large-scale, comparative project that we have undertaken. We anticipate addressing some of the limitations of the present study in continuing collaborative efforts.

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APPENDIX 1.

Taxa, Genbank accession numbers (nr ITS followed by *trn*L-*trn*F; NA = not available), and sources of plant materials from which DNA was extracted for sequencing of the nuclear ribosomal internal transcribed spacer region and chloroplast *trn*L-*trn*F spacer and intron. Abbreviations for herbaria follow Holmgren et al. (1990). Fresh material or material placed directly into silica gel was used except as indicated (HS = herbarium specimen). When plants in cultivation were used, we provide also information on native range. Classification follows results presented herein with regard to Justicieae (with taxa listed alphabetically within lineage), and Manktelow et al. (in press) with regard to others. To facilitate phylogenetic placement of taxa of interest, Appendix 2 lists Justicieae alphabetically and assigns each to a lineage (or grade in the case of Old World "justicioids").

Taxon; Genbank accession number (nr ITS, *trn*L-*trn*F); source and voucher information.

Acanthoideae

*Stenandrium pilosulum* (S. F. Blake) T. F. Daniel; AF169758, AF061827; Mexico. Sonora: Yécora Municipio, El Kipor, *Van Devender & Reina G. 97-434* (ARIZ).

*Aphelandra campanensis* Durkee; AF169760, AF061829; Panama. San Blas: near Mandinga, Río Mandinga, *McDade 852* (DUKE).

Ruellioideae

Ruellieae s.l.

*Sanchezia speciosa* Leonard; AF169385, AF063113; Cultivated, Duke University greenhouses, Durham, North Carolina, U.S.A., Accession No. 66-462 (native to South America), *McDade 1180* (ARIZ).

*Ruellia californica* (Rose) I. M. Johnst.; AF167704, AF063115; Cultivated, Univ. Arizona campus, Tucson, Arizona, U.S.A., *McDade 1157* (ARIZ) (native to northwest Mexico).

Barlerieae

*Barleria lupulina* Lindl.; AF169751, AF289758; Cultivated, San Francisco Conservatory of Flowers, San Francisco, California, U.S.A., *Daniel s.n.* (CAS) (native to Mauritius).

*Lepidagathis villosa* Hedrén; AF169752, AF063121; Scotland et al. (1995) [DNA provided by R. Olmstead (University of Washington) and R. Scotland (Oxford University)].

Justicieae

*Pseuderanthemum* Lineage

*Asystasia gangetica* (L.) T. Anderson; AF289793, AF289748; Cultivated, Nelspruit, Mpumalanga, South Africa, *Daniel & Balkwill 9386* (CAS) (widespread in Old World tropics and subtropics).

*A.* sp.; AF289794, AF289749; Madagascar. Fianarantsoa, Ranomafana National Park, *Daniel 9129* (CAS).

*Chileranthemum pyramidatum* (Lindau) T. F. Daniel; AF289797, AF289752; (HS) Mexico. Chiapas: Municipio La Trinitaria, *Breedlove & Daniel 70767* (CAS).

*Daniel 6737cv*; AF289799, AF289754; (HS) Cultivated, San Francisco Conservatory of Flowers, San Francisco, California, U.S.A., *Daniel 6737cv* (CAS) (native to Madagascar).

*Herpetacanthus stenophyllus* Gómez-Laur. & Grayum; AF289795, AF289750; (HS) Costa Rica. Limón: Reserva Biología Hitoy Cerere, *Herrera 3855* (ARIZ).

*Mackaya bella* Harv.; AF289796, AF289751; Cultivated, Strybing Arboretum, San Francisco, California, U.S.A., *Daniel s.n.* (CAS) (native to southern Africa).

*Odontonema tubaeforme* (Bertol.) Kuntze; AF169748, AF063127; Cultivated, Duke University greenhouses, Durham, North Carolina, U.S.A., Accession No.

66-153, *McDade 1182* (ARIZ) (native to Mexico and Central America).

*Oplonia microphylla* (Lam.) Stearn; AF289798, AF289753; (HS) Cultivated, San Francisco Conservatory of Flowers, San Franciso, California, U.S.A., *Ornduff 7814cv* (CAS) (native to the West Indies).

*Pseuderanthemum alatum* (Nees) Radlk.; AF169749, AF063130; Cultivated, Duke University greenhouses, Durham, North Carolina, U.S.A., Accession No. 84-055, *McDade 1183* (ARIZ) (native to North and Central America).

*Ruspolia seticalyx* (C. B. Clarke) Milne-Redh.; AF289800, AF289755; (HS) Cultivated, Waimea Arboretum and Botanical Garden, Oahu, Hawaii, U.S.A., *Daniel & Butterwick 6635* (CAS) (native to Africa).

*Ruttya fruticosa* Lindau; AF289801, AF289756; Cultivated, San Francisco Conservatory of Flowers, San Francisco, California, U.S.A., *Daniel s.n.* (CAS) (native to Africa).

*Spathacanthus hoffmannii* Lindau; AF289802, NA; (HS) Costa Rica. San José: Reserva Biológica Carara, *Morales 1347* (ARIZ).

*S. parviflorus* Leonard; AF289803, AF289757; (HS) Mexico. Chiapas: El Triunfo Biosphere Reserve, *Daniel et al. 8403* (CAS).

Isoglossinae

*Brachystephanus lyallii* Nees; AF289790, NA; Madagascar. Fianarantsoa, Ranomafana National Park, *Daniel 9101* (CAS).

*Isoglossa grandiflora* C. B. Clarke; AF289788, AF289745; (HS) Cultivated, San Francisco Conservatory of Flowers, San Francisco, California, U.S.A., *Daniel s.n.* (CAS) (native to eastern tropical Africa).

*Isoglossa? sp. 9106*; AF289789, AF289746; Madagascar. Fianarantsoa, Ranomafana National Park, *Daniel 9106* (CAS).

*Ptyssiglottis pubisepala* (Lindau) B. Hansen; AF289787, AF289744; (HS) Papua New Guinea. Madang, ca. 8 km SSW of Madang, *Daniel 6630* (CAS).

*Razisea spicata* Oerst.; AF169848, AF063131; Costa Rica. Heredia: La Selva Biological Station, *Hammel 7974* (DUKE).

*Stenostephanus chiapensis* T. F. Daniel; AF289792, AF289747; Cultivated, San Francisco, California, U.S.A., *Breedlove & Burns 72688cv* (CAS) (native to southern Mexico).

*S. silvaticus* (Nees) T. F. Daniel; AF169747, AF063132; (HS) Costa Rica. San José: Parque Nacional Braulio Carrillo, *Maas 7800* (MO).

Tetramerium Lineage

*Anisacanthus puberulus* (Torr.) Henr. & E. J. Lott; AF289778, NA; Cultivated, Univ. Arizona campus, Tucson, Arizona, U.S.A., *McDade 1179* (ARIZ) (native to southwestern U.S.A. and northern Mexico).

*A. thurberi* (Torr.) A. Gray; AF169846, AF063122; U.S.A. Arizona: Pima County, Tucson Mountains, *Van Devender 88-150* (ARIZ).

*Carlowrightia arizonica* A. Gray; AF169845, AF063123; U.S.A. Arizona: Pima County, Tucson Mountains, *Jenkins 89-24* (ARIZ).

*Chalarothyrsus amplexicaulis* Lindau; AF289780, AF289740; (HS) Cultivated, San Francisco Observatory of Flowers, California, U.S.A., *Daniel & Bartholomew 4842gh* (CAS) (native to western Mexico).

*Ecbolium syringifolium* (Vahl) Vollesen; AF289786, AF289743; (HS) Madagascar. Toliara, Taolanaro, *Daniel & Butterwick 6733* (CAS).

*Fittonia albivenis* (Lindl. ex Veitch) Brummitt; AF289781, AF289741; Cultivated, Univ. Arizona, Tucson, Arizona, U.S.A., *McDade 1178* (ARIZ) (native to South America).

*Gypsacanthus nelsonii* E. J. Lott, V. Jaram. & Rzed.; AF289779, AF289739; Mexico. Puebla: Municipio Coxcatlán, *Daniel 8357* (CAS).

*Henrya insularis* Nees ex Benth.; AF169843, AF063125; Mexico. Sonora: near Alamos, *Jenkins 89-432* (ARIZ).

*Hoverdenia speciosa* Nees; AF289777, AF289738; (HS) Mexico. Hidalgo: Barranca de Tolantongo, *Daniel & Baker 3739* (CAS).

*Mirandea grisea* Rzed.; AF289783, NA; (HS) Mexico. San Luis Potosí: ca. 3 km NW of La Calzada, *Daniel & Baker 3717* (CAS).

*Pachystachys lutea* Nees; AF169844, AF063128; Cultivated, Duke University greenhouses, Durham, North Carolina, U.S.A., Accession No. 84-055 (native to Peru), *McDade 1181* (ARIZ).

*Schaueria calicotricha* (Link & Otto) Nees; AF289782, NA; (HS) Cultivated, Homestead, Florida, U.S.A., *Foote s.n.* (CAS) (native to Brazil).

*Streblacanthus cordatus* Lindau; AF289784, AF289742; Panama. Colón: vicinity of Portobelo, *Daniel et al. 8203* (CAS).

*S. roseus* (Radlk.) B. L. Burtt; AF289785, NA; Cultivated, San Francisco Conservatory of Flowers, San Francisco, California, U.S.A., *Daniel s.n.* (CAS) (native to Peru).

*Tetramerium nervosum* Nees; AF169847, AF063133; U.S.A. Arizona: Pima County, near Patagonia, *McDade & Jenkins 1154* (ARIZ).

Old World "justicioids" (note that the results reported herein indicate that this is not a monophyletic group but rather a grade).

*Anisotes madagascariensis* Benoist; AF289772, AF289733; (HS) Madagascar. Toliara: ca. 20 km N of Toliara, *Daniel & Butterwick 6736* (CAS).

*Duvernoia aconitiflora* A. Meeuse; AF289774, AF289735; Cultivated, Roodepoort, Witwatersrand National Botanic Garden, Gauteng, South Africa, *Daniel et al. 9361* (CAS) (native to southern Africa).

*Justicia adhatoda* L.; AF289773, AF289734; Cultivated, Univ. Arizona campus, Tucson, Arizona, U.S.A., *Barr 60-393* (ARIZ) (native to southern Asia).

*J. betonica* L.; AF289770, AF289731; Cultivated, Johannesberg, Gauteng, South Africa, *Daniel 9369* (CAS) (native to eastern and southern Africa and the Indian Subcontinent).

*J. extensa* T. Anderson; AF289771, AF289732; Cultivated, San Francisco Conservatory of Flowers, San Francisco, California, U.S.A. *Daniel s.n.* (CAS) (native to eastern tropical Africa).

*J. sp. 9024*, AF289768, AF289729; Madagascar. Fianarantsoa, Ranomafana National Park, *Daniel 9024* (CAS).

*J. sp. 9010*; AF289769, AF289730; Madagascar. Fianarantsoa, Ranomafana National Park, *Daniel 9010* (CAS).

*J. sp. 983*; AF289767, AF289728; Madagascar. Masoala Peninsula, Vokoanina Forest, *Zjhra 983* (CAS).

*Metarungia galpinii* (C. Baden) C. Baden; AF289776, AF289737; Cultivated, Johannesberg, Gauteng, South Africa, *Daniel 9322* (CAS) (native to South Africa).

*Rungia klossii* S. Moore; AF289775, AF289736; (HS)

Papua New Guinea. Madang, Bundi, *Daniel et al. 6561* (CAS).

Diclipterinae

*Dicliptera extenta* S. Moore, NA, AF289724; Cultivated, Johannesberg, Gauteng, South Africa, *McDade 1306* (J) (native to southern Africa).

*D. magaliesbergensis* K. Balkwill, NA, AF289725; Witwatersrand National Botanic Garden, Gauteng, South Africa, *Daniel et al. 9357* (CAS).

*D. resupinata* (Vahl) Juss.; AF169841, AF063124; U.S.A. Arizona: Pima County, Santa Catalina Mountains, *Van Devender 84-269* (ARIZ).

*D. suberecta* (André) Bremek.; AF289763, AF289722; Cultivated, Tucson Botanical Garden, Tucson, Arizona, U.S.A., *McDade 1176* (ARIZ) (native to Uruguay).

*D. sp. 9194*; AF289764, AF289723; Madagascar. Fianarantsoa, Ranomafana National Park, *Daniel 9194* (CAS).

*Hypoestes aristata* R. Br.; AF289765, AF289726; (HS) Cultivated, Mildred E. Mathias Botanical Garden, Los Angeles, California, U.S.A., *Daniel s.n.* (CAS) (native to tropical and southern Africa).

*Hypoestes phyllostachya* Baker; AF169842, AF167703; Cultivated, Univ. Arizona, Tucson, Arizona, U.S.A., *McDade 1232* (ARIZ) (native to Madagascar).

*Peristrophe hyssopifolia* (Burm. f.) Bremek.; NA, AF063129; Cultivated, Missouri Botanical Garden, St. Louis, Missouri, U.S.A., Accession No. MBG861410, *MacDougal 5047* (MO) (native to India).

*Rhinacanthus gracilis* Klotzsch; AF289766, AF289727; Cultivated, San Francisco Conservatory of Flowers, San Francisco, California, U.S.A., *Daniel s.n.* (CAS) (native to Africa).

New World Justicioids

*Harpochilus neesianus* Mart. ex. Nees; AF289762, AF289721; (HS) Brazil. Bahia: Santa Luzia, *Souza et al. 5413* (CAS).

*Justicia brandegeana* Wassh. & L. B. Sm.; AF289759, NA; Cultivated, Univ. Arizona campus, Tucson, Arizona, U.S.A., *Starr c.32* (ARIZ) (native to Mexico).

*J. caudata* A. Gray; AF169837, AF063134; Mexico. Sonora: near Alamos, *Faivre 64* (ARIZ).

*J. comata* (L.) Lam.; AF289760, NA; Costa Rica. Heredia: La Selva Biological Station, *Faivre 59* (ARIZ).

*J. longii* Hilsenb.; AF169839, AF063135; U.S.A. Arizona: Pima County, Tucson Mountains, *Van Devender 87-307* (ARIZ).

*J. spicigera* Schltdl.; AF289761, NA; Cultivated, Univ. Arizona campus, Tucson, Arizona, U.S.A., *McDade 1158* (ARIZ) (native to Mexico and Central America).

*Megaskepasma erythrochlamys* Lindau; AF169840, AF063126; Cultivated, Wilson Botanical Garden, Puntarenas, Costa Rica, *McDade 253* (DUKE) (native to northern South America).

*Poikilacanthus macranthus* Lindau; AF169838, AF07066; (HS) Costa Rica. Alajuela Province: Monteverde Reserve, *Haber 707* (MO).

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Mcdade, Lucinda A et al. 2000. "Phylogenetic Relationships within the Tribe Justicieae (Acanthaceae): Evidence from Molecular Sequences, Morphology, and Cytology." *Annals of the Missouri Botanical Garden* 87, 435–458. https://doi.org/10.2307/2666140.

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