

Phylogenetic delimitation of Isoglossinae (Acanthaceae: Justiceae) and relationships among constituent genera.

Carrie A. Kiel¹, Lucinda A. McDade¹, Thomas F. Daniel², & Dominique Champluvier³

¹*Department of Botany, Academy of Natural Sciences, 1900 Ben Franklin Parkway, Philadelphia, Pennsylvania 19103, U.S.A. kiel@acnatsci.org, mcdade@acnatsci.org*

²*Department of Botany, California Academy of Sciences, 875 Howard Street, San Francisco, California 94103, U.S.A. tdaniel@CalAcademy.org*

³*Jardin Botanique National de Belgique, Domaine de Bouchout, B-1860 Meise, Belgium. dominique.champluvier@br.fgov.be*

(summary) Phylogenetic relationships of Isoglossinae (Acanthaceae: Justiceae) were studied with a taxon sample including all but one genus putatively placed in the lineage and a data set composed of DNA sequences from three genic regions (*nrITS*, cp *trnS/G* and *trnT/L*). Two members each of the lineages of Justiceae previously shown to be phylogenetically adjacent to Isoglossinae were included to test monophyly of Isoglossinae. The Malagasy genera *Forcipella* and *Populina* were excluded from Isoglossinae by our analysis. The former was placed outside of *Isoglossinae* and, in fact, outside of Justiceae. The latter was placed with plants representing the *Tetramerium* lineage of Justiceae. Our results do not provide strong support for placement of *Ptyssiglottis* with Isoglossinae but also cannot refute this placement. Core Isoglossinae (i.e., members of Old World *Conocalyx*, *Isoglossa*, *Brachystephanus* and NW *Kalbreyeriella*, *Stenostephanus* s.l., and *Razisea*) are monophyletic; these plants share “Gürtelpollen,” hypothesized to be a morphological synapomorphy for the group. Malagasy *Conocalyx* is part of the clade that includes all sampled species of *Isoglossa* and does not seem distinct from the latter genus. Sister to *Isoglossa* (including *Conocalyx*) is a lineage including all sampled species of

Brachystephanus plus all New World Isoglossinae. Plants belonging to this last clade share monothealous stamens as a morphological synapomorphy. Our data do not support but cannot refute reciprocal monophyly of *Brachystephanus* and NW Isoglossinae. There is, in fact, very little variation among these taxa such that the problem is lack of resolution rather than support for relationships that conflict with current taxonomy. NW plants in particular are richly diverse in morphology and it is remarkable that this diversification is not reflected in variation in these three DNA regions.

KEYWORDS: Acanthaceae, Isoglossinae, Justicieae, *nrITS*, phylogeny, pollen, *trnS/G*, *trnT/L*

INTRODUCTION

Recent phylogenetic work on the large and taxonomically difficult tribe Justicieae (sensu McDade & al., 2000a) has supported the existence of a lineage corresponding to the core of Lindau's (1895) subtribe Isoglossinae. Lindau (1895) used presence of two stamens with mono- or dithealous anthers and "Gürtelpollen" (i.e., girdled pollen; Fig. 1A-D) to distinguish Isoglossinae in his key to tribes and subtribes. Citing the same characters in his treatment of Acanthaceae of Colombia, Leonard (1958) recognized tribe Isoglosseae with two subtribes, Porphyrocominae and Isoglossinae. Lindau's tribe Isoglosseae also likely corresponds in part to Bremekamp's (1965) Rhytiglossinae although Bremekamp placed in the subtribe only Old World plants with dithealous anthers. In any event, Bremekamp did not specify the generic composition of Rhytiglossinae.

Even the limited taxon sampling in phylogenetic work to date, however, indicates that

neither Lindau's Isoglossinae nor Leonard's Isoglosseae were monophyletic as delimited. Both authors assigned to their respective taxa a number of genera that lack the diagnostic traits for the group and some of these have been shown to have closer relatives elsewhere in Justicieae. Lindau's Isoglossinae included New World (NW) *Herpetacanthus* Nees and Old World (OW) *Chlamydacanthus* Lindau. Not surprisingly, the former genus, with plants with four stamens and tricolporate hexapseudocolpate pollen, is a member of the *Pseuderanthemum* lineage of Justicieae (McDade & al., 2000a). The unusual character combination exhibited by plants of *Chlamydacanthus* led Scotland & Vollesen (2000) to treat the genus as unplaced in their synoptic classification of genera of Acanthaceae. *Chlamydacanthus* has since been shown to be closely related to two other OW genera, *Whitfieldia* Hook. and *Lankesteria* Lindl. (Manktelow & al., 2001); Whitfieldieae, as defined by these authors, is not part of Justicieae and may be more closely related to Barlerieae.

Leonard (1958) treated *Fittonia* Coem. and *Megaskepasma* Lindau in his Isoglosseae subtribe Porphyrocominae although plants of these genera lack Gürtelpollen. Our results place these with the *Tetramerium* and NW justicioid lineages of Justicieae, respectively (McDade & al., 2000a). On the other hand, *Razisea*, placed by Lindau and Leonard in Odontoneminae, has two monothealous stamens (Fig. 2F) and Gürtelpollen, and was placed by McDade & al. (2000a) in Isoglossinae. Further, Lindau (1895) placed Old World *Ptyssiglottis* T. Anders. in Pseuderanthemeae whereas the results of McDade & al. (2000a) placed this genus as the basal member of Isoglossinae. Plants of *Ptyssiglottis* have dithealous anthers and a diversity of pollen types. Interestingly, Hansen (1992) treated *Ptyssiglottis* as belonging to Isoglossinae but without explicit basis for doing so. The phylogenetic placement of *Ptyssiglottis* as part of Isoglossinae merits further study as this result was not strongly supported in the analyses presented by

McDade & al. (2000a). Table 1 summarizes the putative members of Isoglossinae and provides data on species richness and geographic range.

Despite progress in delimiting Isoglossinae, phylogenetic work to date has been based on limited taxon sampling. Our earlier work included only three species of NW and four of OW Isoglossinae. Among genera assigned by Lindau (1895) to Isoglossinae, *Forcipella* Baill., *Oreacanthus* Benth., and *Populina* Baill. (also *Strophacanthus* Lindau, treated as congeneric with *Isoglossa* Oerst. by Scotland & Vollesen, 2000) were not included. More recently described genera that would appear to belong to Isoglossinae based on morphological characters include *Conoclayx* Benoist and *Sphacanthus* Benoist; these have also not yet been placed phylogenetically. Generic delimitations have also been in flux and contrasting taxonomic hypotheses merit phylogenetic study. Champluvier (in prep.) has recently monographed *Brachystephanus* Nees to include *Oreacanthus* and comprise three subgenera. Clearly, the single species of *Brachystephanus* included in our previous work did not permit phylogenetic evaluation of these taxonomic changes. New World Isoglossinae have been treated as belonging to a number of poorly delimited genera, including *Cylindrosolenium* Lindau, *Habracanthus* Nees, *Hansteinia* Oerst., *Kalbreyeracanthus* Wassh., *Kalbreyeriella* Lindau, *Razisea* Oerst., *Stenostephanus* Nees, and *Syringidium* Lindau. Focussing on Colombian plants, Wood (1988) examined the characters that have been used to distinguish *Habracanthus*, *Hansteinia* and *Kalbreyeracanthus* and concluded that the three genera could not be clearly delimited. As a result, Wood (1988) treated *Hansteinia* and *Kalbreyeracanthus* as synonyms of *Habracanthus*. More recently, *Cylindrosolenium*, *Habracanthus*, *Hansteinia* and *Syringidium* have all been treated as part of *Stenostephanus* (e.g., Daniel, 1995, 1999; Wasshausen & Wood, 2001). Daniel (1999) also questioned whether other New World genera of Isoglossinae (i.e., *Kalbreyeriella* and

Razisea) were sufficiently morphologically distinct from *Stenostephanus* to warrant maintaining them. Our earlier sample was inadequate to address the validity of these taxonomic modifications and queries.

With a nearly complete sample at the generic level and multiple species to represent most genera and subgeneric taxa, we have studied Isoglossinae using data from DNA sequences and morphology to address the following questions. (1) Are genera traditionally placed in Isoglossinae or predicted to be part of the lineage based on morphological characters (i.e., those listed in Table 1) indeed part of this lineage and are their morphological characters consistent with phylogenetic relationships? (2) With denser taxon sampling and data from additional genic regions, is *Ptyssiglottis* supported as the basal lineage of Isoglossinae? (3) Are the OW and NW members of Isoglossinae reciprocally monophyletic? Our earlier work (McDade & al., 2000a) suggested that NW Isoglossinae are monophyletic whereas OW Isoglossinae are paraphyletic, but taxon sampling was inadequate. (4) Are currently recognized OW and NW genera and subgeneric taxa monophyletic? Table 2 presents these and other ideas based on morphological characters as hypotheses to be tested.

We have also expanded the character basis for this phylogenetic work substantially. Previous research on Isoglossinae included sequences from the nuclear ribosomal internal transcribed spacer, *nrITS*, and the chloroplast *trnL/F* region (i.e., including intron, exon and spacer). The *trnL/F* region showed very little variability among Isoglossinae and we have instead gathered sequences from the more rapidly evolving *trnT/L* and *trnS/G* spacers, as well as *nrITS*.

MATERIAL AND METHODS

Taxon Sampling.---We obtained sequences for species representing all of the genera placed by Lindau (1895) in Isoglossinae except those shown to have relationships elsewhere, as described above, and *Sphacanthus*. DNA was not available for this last poorly known genus and collections of it are few. Also included were genera that have been shown to be part of Isoglossinae (*Ptyssiglottis*; McDade & al., 2000a) or are thought to be part of Isoglossinae based on morphology (*Conocalyx*). To test monophyly of Isoglossinae and determine its constituents, we included members of the lineages that were shown to be adjacent to Isoglossinae by McDade & al. (2000a): the *Pseuderanthemum* (*Odontonema tubaeforme*, *Pseuderanthemum floribundum*) and *Tetramerium* (*Ecbolium syringifolium*, *Tetramerium wasshausenii*) lineages. Because one of our goals was to test monophyly of Old World *Brachystephanus*, we included species that represent the three recognized subgeneric taxa (Champluvier, in prep.). Likewise, among New World Isoglossinae, we included species that have been treated as *Habracanthus*, *Kalbreyeriella*, *Razisea* and *Stenostephanus*. Distinctions among these genera are subtle to lacking, and current practice is to treat some or all as members of *Stenostephanus* (e.g., Daniel, 1995) but the process of detailed study and nomenclatural renovations remain incomplete. Two species of *Crossandra* (Acantheae) were used as out-groups for the purpose of rooting our phylogenetic hypotheses. Appendix 1 lists taxa included here along with voucher and sources of material used.

Molecular Methods.---Fresh leaf material, leaf material dried in silica gel or from recently collected herbarium specimens that appeared to be in good condition were used as sources of DNA. Genomic DNA of silica dried or fresh material was extracted using the modified CTAB method of Doyle & Doyle (1987). For material from herbarium specimens, we used QIAGEN DNEasy™ kits for DNA extraction because the kits produced higher yields of higher molecular

weight DNA than bulk methods.

Procedures for purifying genomic DNA and for amplifying the *nrITS* and *trnS/G* regions were as described by McDade & al. (2000b) and McDade & al. (2005), respectively. The *trnT/L* spacer was amplified using the 5' *trnT* exon ("a") and 3' *trnL* exon ("b") primers of Taberlet & al. (1991). As noted by Shaw & al. (2005), these primers do not as reliably amplify plant DNAs as the other Taberlet primers; they successfully amplified only about half of Acanthaceae DNAs attempted. We thus designed a primer slightly internal to the "b" primer (ACA3'*trnT/L*: 5'TTTNACTNAAACCCTTGAAT). Interestingly, the 5' end of the *trnT/L* intron is highly variable in Acanthaceae such that, even with a number of sequences in hand, it was not feasible to design a new internal primer at the 5' end. Amplification with the primer pairs "a" and ACA3'*trnT/L* was successful for the Isoglossinae DNAs that had failed to amplify with the original Taberlet primers. PCR products were purified using QIAGEN QiaQuick™ PCR purification kits.

Sequences were generated on a Beckman Coulter capillary sequencer using the same primers as in amplification except that, for *trnT/L*, ACA3'*trnT/L* was almost always used in sequencing even when the Taberlet "b" primer was used for PCR. For most samples, sequences were read in both directions for verification and to complete the sequence. In particular, the *trnS/G* sequences for Isoglossinae had a poly-A/T string 7-18 bases long that usually could not be read through; thus, both forward and reverse sequences were necessary to verify the number of Ts at that position and to complete the sequence. Electropherograms of all sequences were proofread manually. Overlapping portions were reconciled by reverse-complementing one, aligning both, and double-checking any inconsistencies against the electropherograms;

mismatches that could not be resolved were coded as uncertain.

Alignments and Analysis.---Sequences for each DNA region were aligned separately by eye in SeqApp 1.9a169 (Gilbert, 1992). Sequences were easily aligned across the sampled taxa except that short portions of *nrITS* and *trnS/G* could not be aligned unambiguously across the outgroup and Justiciaeae sequences. These were scored as missing for the out-group taxa. As noted by a number of authors (e.g., Gielley & al., 1996; Kim & al., 1996; McDade & Moody, 1999), the non-coding chloroplast regions have a relatively high frequency of parsimony informative indels (i.e., length mutations). Thirteen and three indels were added to the *trnS/G* and *trnT/L* data matrices, respectively, as presence/absence characters. No indels were added to the *nrITS* data set. The indels were identified conservatively (i.e., only when they had common 5' and 3' termini across all relevant taxa) and were parsimony informative. Data matrices of the three DNA regions were prepared as Nexus files in MacClade version 4.0 (Maddison & Maddison, 2000). Table 3 compares the three genic regions in terms of length, variability, number of taxa sequenced and missing data. We were unable to obtain *nrITS* sequences for five taxa, presumably due to low quality DNA from herbarium specimens. In addition, *trnS/G* and *trnT/L* data are missing for one taxon each (Appendix 1); these failed to amplify or to give clean sequence data despite repeated attempts.

The three data sets were tested for congruence using the partition homogeneity test (implemented in PAUP* [Swofford, 2000] as the Incongruence Length Difference test; 100 replicates, 25 random addition sequences, maxtrees = 20,000). For each pairwise comparison of DNA regions, the data sets were first pruned to include only taxa for which both of the relevant loci were available. Not surprisingly, the two cp spacer regions were congruent ($P = 0.56$) and

these data sets were combined. The ILD test indicated incongruence between the cp and *nrITS* data ($P = 0.01$) but the sums of tree lengths from the 100 resampled data sets ranged from only two to 13 steps (0.2--1.4%) longer than the sum of tree lengths from the original partition. The source of incongruence was determined by inspecting the results from parsimony analyses (in PAUP*) of the cp versus nuclear data to identify taxa placed differently. Two species of *Isoglossa* were placed with *Odontonema* + *Pseuderanthemum* by the nuclear data and with other species of *Isoglossa* by the cp data. However, the placement of these two species was very weakly supported by the *nrITS* data (e.g., bootstrap support for all relevant branches was $< 50\%$) and a Templeton test indicated that the *nrITS* data could not reject placement of these two species with other *Isoglossa* ($P = 0.23$). Further, the data sets were congruent when these taxa were removed or when *nrITS* data were scored as missing for them ($P = 0.39$). In our previous work with other Acanthaceae, we have encountered similar instances of highly divergent *nrITS* sequences (McDade & al., 2000b; see also Buckler IV & Holtsford, 1996; Alvarez & Wendel, 2003). We thus combined the nuclear and cp data sets after scoring the *nrITS* data as missing for the two species with aberrant sequences. We present results from analysis of this combined data set.

All parsimony analyses used rigorous heuristic search strategies designed to find all islands of equally parsimonious trees (i.e., multiple random addition sequences and TBR branch swapping). Strength of support for individual branches in the parsimony trees was evaluated using nonparametric bootstrap values (BS; Felsenstein, 1985) and decay indices (DI; Bremer, 1988; Donoghue & al., 1992). Bootstrap values are from 100 replicates with 25 random addition sequences and TBR branch swapping; for some analyses, maxtrees was set to 50,000. DIs for each branch were determined by first using MacClade to prepare a “Decay Index PAUP file”

from the strict consensus of most parsimonious (MP) trees. This file was then executed in PAUP* to find the shortest trees inconsistent with each branch of the strict consensus tree; settings for these searches were as described above. The difference between the length of these trees and the globally shortest trees is the decay index (DI) for the branch in question.

Bayesian likelihood analyses were done in MrBayes v3.0B4 (Huelsenbeck & Ronquist, 2001; Huelsenbeck & al., 2001; Ronquist & Huelsenbeck, 2003), run with settings corresponding to a GTR model with gamma-distributed rate variation and proportion of invariant sites estimated by the program. Three heated and one ‘cold’ chain were run for > 1,000,000 generations, with trees saved every 100 generations. Analysis of the combined cp and nuclear data used a mixed model approach, permitting the model of evolution to be optimized independently for data from the two genomes. Bayesian posterior probability values for branches were determined by opening the tree file produced by MrBayes in PAUP, filtering to remove the pre-burn-in trees from consideration, and then constructing the majority rule consensus tree; this tree is the maximum a posteriori tree (MAP).

When the results did not permit clear, strongly supported answers to our questions about Isoglossinae relationships, as enumerated in Table 2, we used MacClade to prepare trees reflecting the relationships of interest. These were loaded into PAUP* as constraint trees using the same search strategy described above except that PAUP* was asked to find the shortest trees consistent with the constraints. One of the resultant MP trees consistent with each of the constraints was randomly selected and compared to one randomly selected MP tree using Templeton’s test in PAUP* (reported as z statistics). The same strategy was used to compare likelihood scores of trees reflecting alternative phylogenetic hypotheses with all likelihood

parameters (except base frequencies for which empirical values were used) estimated using one randomly selected MP tree. These parameters were then used as the model to compare likelihood scores of the most likely tree to that of trees consistent with each of the alternative phylogenetic hypotheses using the Kishino-Hasegawa RELL test (K-H RELL) as implemented in PAUP*. Tests were one-tailed because an optimal tree was one of the trees being compared in each case (Felsenstein, 2004: 369).

RESULTS

Molecular Evolution.---Table 3 compares the genic regions used in our analysis in terms of variability, homoplasy and missing data. The *nrITS* region has more parsimony informative characters than either of the cp regions (*nrITS* = 99, *trnS/G* = 91, *trnT/L* = 56). However, length mutations augmented the phylogenetic signal provided by the cp regions, most notably the *trnS/G* spacer; in contrast, no informative indels were scored for *nrITS*. Of the two cp loci, the *trnS/G* spacer is considerably more variable, both in terms of substitutions and length mutations. Notably, in terms of pairwise distances between taxa, the *trnS/G* spacer nearly matches *nrITS*. The combined data set had a total of 246 parsimony informative characters plus 16 informative length mutations.

Phylogenetic Relationships.---To our surprise, *Forcipella* was placed outside of Isoglossinae by sequence data from all three loci; in fact, it was not associated with any of the Justiceae included in our analysis (results not shown). Because the data sets assembled for Isoglossinae do not include enough taxa beyond Justiceae to place *Forcipella* with confidence, we excluded it from the present analysis and will place this taxon in a future study involving a more comprehensive sample of Acanthaceae.

Maximum parsimony and Bayesian likelihood analyses yielded congruent results for all aspects of relationship that were supported by bootstrap values > 70% or posterior probabilities \geq 85%. Fig. 3 presents the Bayesian maximum a posteriori (MAP) tree including branches with \geq 85% posterior probability. Presented below the Bayesian posterior probability values are bootstrap and decay index values from parsimony analyses. Fig. 4 presents one randomly chosen MP tree to illustrate branch lengths.

Our analysis placed *Populina* outside of Isoglossinae and within the *Tetramerium* lineage with strong support (Bayesian posterior probability, BPP = 100; bootstrap, BS = 100; decay index, DI = 21; Fig. 3). Although taxon sampling within the *Tetramerium* lineage is extremely sparse in the data sets analyzed here, it is interesting that *Populina*, native to Madagascar, is placed sister to Old World *Ecbolium*, with NW *Tetramerium* sister to these together. The relationships of *Populina* will be examined further in future work with more comprehensive sampling of the *Tetramerium* lineage.

The monophyly of Isoglossinae including *Ptyssiglottis* is weakly supported by the Bayesian likelihood analysis (BPP = 85), but the lineage is not present in all of the MP trees (e.g., the randomly selected MP tree presented as Fig. 4 places *Ptyssiglottis* with the *Tetramerium* lineage). Based on our limited sampling of *Ptyssiglottis* (i.e., 2 of 33 species; Table 1), our results indicate that the genus is monophyletic with very strong support (BPP, BS, DI = 100, 100, 16).

Core Isoglossinae (i.e., *Isoglossa*, *Conocalyx*, *Brachystephanus*, and all NW Isoglossinae) are monophyletic with very strong support (BPP, BS, DI = 100, 100, 25; note the

relatively long branch to this clade in Fig. 4). There is strong Bayesian and moderate parsimony support (BPP, BS, DI = 100, 68, 3) for a lineage that includes *Conocalyx* and all sampled species of *Isoglossa*. Within this lineage, two species of *Isoglossa*, *I. stipitata* and *I. ciliata*, are close relatives with strong support (BPP, BS, DI = 100, 96, 3). Other aspects of relationships among *Isoglossa* are not as strongly supported.

There is strong support from both Bayesian likelihood and parsimony for a lineage that includes all sampled species of OW *Brachystephanus* and NW Isoglossinae (BPP, BS, DI = 100, 100, 13). Not surprisingly given this last result, monophyly of OW Isoglossinae (i.e., all sampled species of *Isoglossa*, *Conocalyx*, *Brachystephanus*) is rejected by our data (Templeton test, $P < 0.001$; K-H RELL, $P < 0.05$; Table 2, Hypothesis 3).

Our analysis indicates that the Old World genus *Brachystephanus* (sensu Champluvier, in prep.) is paraphyletic: sampled species are placed as a series of lineages basal to or within the clade that comprises all sampled NW species. Among the sampled *Brachystephanus*, six species are resolved in pairs with strong (*B. africanus* + *B. lyallii*), moderate (*O. manni* + *B. sp. nov. 1*), and relatively weak support (*B. manni* + *O. montifuga*). The seventh species, *B. sp. nov. 2*, is not closely associated with other species of *Brachystephanus*. Despite the fact that *Brachystephanus* is paraphyletic in both the parsimony and Bayesian likelihood results, our data cannot reject a monophyletic *Brachystephanus* (Table 2, Hypothesis 8).

Our data do not resolve relationships among NW Isoglossinae except that the small, mostly Central American genus *Razisea* is monophyletic (BPP, BSS, DI = 100, 100, 5). There is no indication of a lineage corresponding to *Habracanthus*, but this may be due to very little

variability among these taxa (note short branch lengths among these taxa in Fig. 4).

DISCUSSION

Regarding relative variability of the two cp regions, and thus their utility at low taxonomic levels, our results concur with the data reported by Shaw & al. (2005) in that the *trnS/G* spacer has considerably more informative characters than *trnT/L*. This cp region should thus be more useful at lower taxonomic levels than other regions of the chloroplast genome that have been commonly used in phylogenetic studies. This was true also for indels, with *trnS/G* contributing 13 of 16 informative indels. One drawback of *trnS/G* for these plants was the presence of a polyA/T string up to 18 bases in length about 350 aligned bases from the *trnS* priming site. When sequences had more than 12-14 A/Ts at this location, we were unable to sequence through it.

Our results clearly exclude *Forcipella* and *Populina* from Isoglossinae. Both were placed by Lindau (1895) in the subtribe, but plants of these genera have at least some characters that are not at home there. Although *Forcipella* has pollen that appears to be Gürtelpollen (Muller & al., 1989; Daniel, Kiel, McDade pers. obs.), the plants have four ditheous stamens whereas Isoglossinae have two di- or monothecous stamens. We will include *Forcipella* in future research that seeks to place phylogenetically a number of acanth genera of uncertain affinities. The androecium of *Populina* (i.e., two ditheous stamens) is not out of place in Isoglossinae but plants of this genus have tricolporate hexapseudocolpate pollen (Muller & al., 1989). Our results place *Populina* with the *Tetramerium* lineage with very strong support; future work will address the phylogenetic placement of this genus more precisely.

Our data strongly support monophyly of *Ptyssiglottis* but we are not yet able to place the genus with confidence. Assessment of the relationships of this genus requires either more data or more comprehensive taxon sampling, or both. Species of *Ptyssiglottis* have two ditheous stamens (Fig. 2A) (staminodes have been observed in one species, Hansen, 1992), but they have pollen types other than Gürtelpollen (Hansen, 1992; Fig. 1E,F). In fact, Hansen's (1992) studies of pollen in 32 of the 33 species that he recognized in the genus (pollen of *P. longisepala* B. Hansen is unknown) showed that Gürtelpollen does not occur in *Ptyssiglottis*. Scotland & Vollesen's (2000) report of Gürtelpollen from the genus is for *Ptyssiglottis terminalis* (Fawc.) Moore, a species that was referred to *Isoglossa dichotoma* (Hassk.) B. Hansen by Hansen (1992). *Ptyssiglottis* thus cannot be strongly associated with Isoglossinae based either on morphological or molecular evidence.

Our data provide very strong support for 'core Isoglossinae' (i.e., *Isoglossa*, *Conocalyx*, *Brachystephanus* and all NW Isoglossinae). We propose that Gürtelpollen evolved in the common ancestor of this lineage and is synapomorphic for it. There are, however, two potential problems with this hypothesis. First, in his treatment of Asian *Isoglossa*, Hansen (1985) included species with both Gürtelpollen and tricolporate hexapseudocolpate pollen (i.e., like those of the *Ptyssiglottis* species illustrated in Fig. 1E). Our results predict that, if species with this last pollen type are indeed Isoglossinae, they will be placed basal to the lineage referred to here as "core Isoglossinae." Unfortunately, all material of these species available to us is on herbarium sheets at least 70 years old and we were not able to extract usable DNA from any despite multiple attempts. Second, we note again that pollen of *Forcipella* and *Whitfieldieae* (sensu Manktelow & al., 2001) is remarkably similar to Gürtelpollen. Based on TEM study of a small sample of taxa,

C. A. Furness (pers. comm.) suggests that pollen of Whitfieldieae and core Isoglossinae can be distinguished only by wall structure. Pollen of *Whitfieldia* has a homogeneous granular ectexine whereas grains of *Isoglossa* have distinct columellae which may be branched. Given the extreme pollen diversity encompassed by Acanthaceae, it is perhaps not surprising to document instances of parallel evolution of similar pollen. McDade & al. (2005) have likewise proposed that there has been parallel evolution of a remarkably distinctive pollen type in Acantheae.

Among core Isoglossinae, plants of *Conocalyx* and *Isoglossa* have ditheous stamens (Fig. 2B) whereas plants of all other genera have monotheous stamens (Fig. 2C-F). Our results place *Conocalyx* and *Isoglossa* basal to other Isoglossinae which is consistent with the hypothesis that ditheous stamens are plesiomorphic and monotheous stamens are apomorphic. The stamens of plants of *Isoglossa* have thecae that are uneven in height and often divergent (i.e., set at markedly different angles on the filament and connective; Fig. 2B). It is tempting to hypothesize that these androecial modifications set the stage evolutionarily for the loss of one theca. On the other hand, such androecial modifications are nearly universal among ‘justicioids’ (sensu McDade & al., 2000a) yet none of these plants have lost one of the thecae entirely.

Conocalyx is placed by our analysis as part of a clade that includes all sampled species of *Isoglossa*. In Benoist’s (1967) description of *Conocalyx* as a new genus with a single species, he did not indicate how it is distinguished from *Isoglossa*. Like *Isoglossa*, the plants have two ditheous stamens, no staminodes, and Gürtelpollen (Muller & al., 1989). *Conocalyx* is clearly not phylogenetically distinct from species placed in *Isoglossa* and, given the morphological diversity encompassed by *Isoglossa*, *Conocalyx* does not seem morphologically distinctive either.

All sampled species of *Isoglossa* (and *Conocalyx*) form a clade. However, it is important to note that our sample does not yet include enough species of the large (ca. 50 species) genus *Isoglossa* to constitute a thorough study of the group. Notably, we have under-sampled species from tropical Africa and Madagascar, and, as described above, we were not able to obtain DNA of any of the eight Asian *Isoglossa*. Expanding the sample to include two or three species from each of these areas would constitute a stronger test of monophyly of *Isoglossa*.

All sampled species of *Brachystephanus* and of NW Isoglossinae are together monophyletic with strong support in our analysis. So far as is known, these plants all have monothealous anthers (Fig. 2C-F) and we hypothesize that this trait evolved in the common ancestor of this lineage and represents a synapomorphy for the group. These plants also have Gürtelpollen which is a symplesiomorphy at this level if our interpretation of this character as having evolved in the common ancestor of all core Isoglossinae is correct.

Neither *Brachystephanus* nor NW Isoglossinae is monophyletic in our analyses (Fig. 3), but our data cannot reject reciprocal monophyly of these groups. As indicated by the very short branch lengths among these taxa in Fig. 4, there are few data to support any resolution of these lineages and more data or data from a more rapidly evolving locus will be necessary to provide a rigorous test of monophyly of these taxa. Daniel (1999) and Champluvier (in prep.) have both discussed problems with the characters traditionally used to distinguish *Brachystephanus* from NW Isoglossinae. Based on detailed study of flowers of several species, Champluvier proposed that *Brachystephanus* and *Stenostephanus* may be distinguished by point of insertion of filaments. In flowers of the species of *Stenostephanus* that she studied, the filaments are inserted at or below the mid-point of the corolla tube whereas, in *Brachystephanus*, filaments are inserted near the mouth of the corolla. This character holds for a number of other species of NW

Isoglossinae including three species of *Razisea* (*R. spicata*, *R. wilburii*, *R. villosa*; L. McDade, unpubl. data), but it does not seem to be consistent. Daniel (1999) described Mexican *Stenostephanus* as having the filaments inserted near the apex of the corolla tube or near the base of the throat and Wasshausen & Wood (2001) describe *S. tenellus* as having filaments inserted about 2/3 from the base of the corolla tube. This and other characters that may distinguish these plants require further study. Given that our results indicate that the OW and NW plants may not be reciprocally monophyletic, it would not be surprising if unambiguous differences could not be found.

The species of *Brachystephanus* form a series of lineages that are basal to or part of the large polytomy that also includes all NW Isoglossinae. Champluvier (in prep.) studied both *Brachystephanus* and *Oreacanthus* and concluded that they are best treated as a single genus. Our results concur in that the two sampled species that had been treated in *Oreacanthus*, *O. montifuga* and *O. mannii*, are not each other's closest relatives. Morphologically, two of Champluvier's infrageneric taxa, "Brachystephanus" and "Oreacanthus" differ by the shape of the stigma, the form of the lips of the corolla, and morphology of pollen grains. The third, "Pseudoreacanthus" is purported to be intermediate between "Brachystephanus" and "Oreacanthus," based on having a stigma like that of "Brachystephanus," a corolla like that of "Oreacanthus," and pollen largely intermediate between these infrageneric taxa. Our results provide support for Champluvier's (in prep.) "Brachystephanus," in that the two sampled species, *B. africanus* and *B. lyallii*, are each other's closest relatives, and for "Pseudoreacanthus," the sole species of which, *B. sp. nov. 2* is not closely related to any of the other sampled species of *Brachystephanus*.. On the other hand, "Oreacanthus" may not be monophyletic. The four sampled species are placed as the pairs *O. montifuga* + *B. mannii* and *B. gigantiflora* + *B. sp. nov. 1* but these four species are not together

monophyletic.

All sampled NW Isoglossinae are part of a polytomy that also includes at least two lineages of *Brachystephanus*. Despite this, as discussed above, our data provide too few characters to refute monophyly of NW Isoglossinae. Among NW plants, there is a remarkable diversity of floral morphology; corolla tubes range in length from < 1cm to > 6 cm and may be white, pale blue to purple, yellow, orange, red or even bicolored (Fig. 2D-F). Despite this diversity, as pointed out by a number of authors, the morphological characters cited as distinguishing genera of NW Isoglossinae do not consistently do so (Wood, 1988; Daniel, 1999; Wasshausen & Wood, 2001). Our results likewise provide no basis for recognizing multiple genera in the NW with the possible exception of *Razisea*, the two included species of which were monophyletic with strong support. Species of *Razisea* share distinctive corolla morphology: the lobes of the lower lip are extremely short such that the tube truncates abruptly (Fig. 2F). It must be noted, however, that with regard to our molecular data, the problem is one of lack of resolution rather than of support for a series of NW lineages than blend traditional genera. Thus, whereas these data do not support the recognition of traditional genera, they do not provide a strong refutation of such taxa. Sequence data from a more rapidly evolving locus (or simply many more characters) will be necessary to achieve strongly supported resolution among NW Isoglossinae. It is also possible that the lineage radiated rapidly in the NW leaving very short branches among these taxa that will be difficult to reconstruct.

Cytologically, Isoglossinae remain one of the least known lineages of Acanthaceae. Based on chromosome numbers reported to date, a few patterns are evident among the basal sublineages of Justiceae used in this study (i.e., the *Pseuderanthemum* and *Tetramerium* lineages

in addition to Isoglossinae; lineage names as in McDade & al., 2000). The *Pseuderanthemum* lineage is characterized by numerous counts of $n = 21$, and a base number of $x = 21$ has been postulated for that lineage (Daniel, 2000; McDade & al., 2000a). The *Tetramerium* lineage, which is distal to Isoglossinae in Justicieae (McDade & al., 2000a), is characterized by many counts of $n = 18$, and a base number of $x = 18$ is likely for that lineage (Daniel, 2000; McDade & al., 2000a). Within Isoglossinae, there are counts for three species of the Old World genus *Isoglossa*; all of them are $n = 17$ (Daniel & al., 2000). All counts for New World representatives of Isoglossinae are $n = 18$ (i.e., *Stenostephanus* [including taxa treated as *Hansteinia*] and *Razisea*; Daniel, 1999). These numbers suggest that the common ancestor of Justicieae distal to the *Pseuderanthemum* lineage might have had $n = 18$, and that an aneuploid reduction from 18 to 17 occurred in the OW lineage of Isoglossinae that includes plants with dithecous anthers (including most species of *Isoglossa*). No counts have been made for Malagasy species of *Isoglossa*. In this scenario, the clade characterized by the synapomorphy of a single anther theca retained the primitive chromosome number of $n = 18$. Chromosome counts have not been obtained for any species of *Brachystephanus* but the proposed scenario predicts $n = 18$ for these plants.

In sum, our results confirm that Isoglossinae are a distinct lineage, although whether *Ptyssiglottis* is part of the clade remains uncertain. We suggest that the group is worthy of taxonomic recognition, although we do not intend to propose a rank pending revised classification of Justicieae as a whole. Notably, as we have shown for Acantheae (McDade & al., 2005), OW and NW members of Isoglossinae are not reciprocally monophyletic; instead OW plants form a series of lineages basal to and including NW Isoglossinae. The inability of our molecular data to resolve relationships among NW Isoglossinae and, indeed, among members of

the monothecous clade of core Isoglossinae (i.e., NW Isoglossinae + *Brachystephanus*) is mirrored by the confused taxonomic history of the group at the genus level. This confusion in turn reflects the lack of clear morphological characters that would permit unambiguous delimitation of genera. It remains to be seen whether this low signal from the molecular data can be overcome by gathering more data or whether the present result accurately reflects the evolutionary history of the group. Inferences from biogeography suggest that more data may help. Our results clearly point to an OW origin for Isoglossinae. As the origin of Acanthaceae, and thus certainly of relatively distal lineages like Isoglossinae, likely post-dates Gondwanaland (see McDade & al., 2005 for discussion of relevant fossil data regarding the age of Acanthaceae), we hypothesize that long-distance dispersal is responsible for presence of the lineage in the NW. These plants have ballistic seed dispersal that is on the order of meters to tens of meters. Thus, more than one dispersal event from the Old to the New World seems unlikely. We therefore hypothesize that NW Isoglossinae are monophyletic and it should be possible to resolve at least that relationship with confidence with additional data.

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Appendix 1. Taxon; Location; Voucher; Genbank accession numbers *trnS/G*, *trnT/L*, *nrITS* (NA = not available); (OG) indicates taxa used as out-groups; (Other Justiciaeae) indicates taxa belonging to lineages adjacent to Isoglossinae.

Brachystephanus africanus S. Moore; Tanzania, *Luke et al. 6704* (US); DQ372491, DQ372446, DQ372469. *Brachystephanus lyallii* Nees; Madagascar, *Daniel et al. 9101* (CAS); DQ372492, DQ372447, AF289790. *Brachystephanus mannii* C.B. Clarke; Cameroon, *Cheek 10287* (BR); DQ372493, DQ372448, DQ372470. *Brachystephanus* sp. nov.1; Cameroon (Mt. Kupe.), *Lane 186* (BR); DQ372497, DQ372452, DQ372472. *Brachystephanus* sp. nov. 2; Cameroon, *Letouzey 13412* (BR); DQ372496, DQ372451, NA. *Conocalyx laxus* Benoist; Madagascar, *Leandri 3033* (P); DQ372485, DQ372440, NA. (OG). *Crossandra greenstockii* S. Moore; South Africa, *McDade & Balkwill 1241* (J); DQ059250, DQ372463, DQ028427 (OG). *Crossandra longipes* S. Moore; Madagascar, *Hearn Mad-62* (PH); DQ059253, DQ372464, NA. (Other Justiciaeae) *Ecbolium syringifolium* (Vahl) Vollesen; Madagascar, *Daniel & Butterwick 6733* (CAS); DQ372480, DQ372435, AF289786. *Forcipella* sp.; Madagascar, *Daniel et al. 10432* (PH); results reported here place this plant outside of Isoglossinae and of Justiciaeae, sequences will be reported in a subsequent paper. *Habracanthus charien* Leonard; Colombia, *Wood 4547* (US); DQ372503, DQ372458, DQ372476. *Habracanthus macrochilus* Lindau; Colombia, *Wood 4977* (US); DQ372499, DQ372454, DQ372474. (Note that *Stenostephanus haematodes* (Nees) T. F. Daniel and *S. silvaticus* (Nees) T. F. Daniel were originally treated as *Habracanthus*.) *Isoglossa ciliata* Oerst.; South Africa, *Balkwill et al. 11653* (PH); DQ372489, DQ372444, NA. *Isoglossa gracillima* Baker; Madagascar, *Daniel 9106* (CAS); DQ372488, DQ372443, AF289789. *Isoglossa grandiflora* C. B. Clarke; Cultivated, *Daniel s.n.* (CAS); DQ372490, DQ372445, AF289788. *Isoglossa ovata* (Nees) Lindau; South Africa, *Daniel 9336* (CAS); DQ372487, DQ372442, DQ372468. *Isoglossa stipitata* C.B. Clarke; South Africa, *Daniel 9366* (CAS); DQ372486, DQ372441, DQ372467. *Kalbreyeriella rostellata* Lindau;

Colombia, *McDade 1007* (DUKE); DQ372498, DQ372453, DQ372473. (Other Justiceae)

Odontonema tubaeforme (Bertol.) Kuntze; cultivated, *McDade 1182* (ARIZ); DQ059297, DQ372462, AF169748. *Oreacanthus mannii* Benth.; Cameroon, *Leeuwenberg 8925* (BR); DQ372495, DQ372450, DQ372471. *Oreacanthus montifuga* Milne-Redhead; Democratic Republic of Congo, *Quarre 5622* (BR); *Populina richardii* Baill; Madagascar, *Kerardren 1671* (P); DQ372482, DQ372437, NA (results reported here place this plant in the *Tetramerium* lineage of Justiceae rather than with Isoglossinae). (Other Justiceae) *Pseuderanthemum floribundum* (Oerst.) Leonard; cultivated, *Daniel 5381cv* (CAS); DQ372507, NA, DQ372479. *Ptyssiglottis psychotriifolia* (Stapf) B.Hansen; Borneo, *Poulsen 40* (C); DQ372484, DQ372439, DQ372466. *Ptyssiglottis pubisepala* (Lindau) B.Hansen; Papua New Guinea, *Daniel 6630* (CAS); DQ372483, DQ372438, AF289787. *Razisea citrina* D.N. Gibson; Costa Rica, *Hammel 19242* (DUKE); DQ372501, DQ372456, NA. *Razisea spicata* Oerst; Costa Rica, *Hammel 7974* (DUKE); DQ372502, DQ372457, AF169848. *Stenostephanus chiapensis* T. F. Daniel; Mexico, *Breedlove & Burns 72688* (CAS); DQ372506, DQ372461, AF289792. *Stenostephanus haematodes* (Nees) T. F. Daniel; Mexico, *Ventura 4670* (ARIZ); DQ372500, DQ372455, DQ372475 (originally described in *Habracanthus*). *Stenostephanus krukoffii* Wassh.; Bolivia, *Wood 13915* (US); DQ372504, DQ372459, DQ372477. *Stenostephanus lobeliiformis* Nees; Brazil; *Wasshausen 2350* (US); DQ372505, DQ372460, DQ372478. *Stenostephanus silvaticus* (Nees) T. F. Daniel; Costa Rica, *Maas 7800* (MO); NA, NA, AF169747 (originally described in *Habracanthus*). (Other Justiceae) *Tetramerium wasshausenii* T. F. Daniel; Peru, *Jenkins PJ00-170* (ARIZ); DQ372481, DQ372436, DQ372465.

Table 1. Genera, species richness and range of Old World and New World genera associated with Isoglossinae. Total number of species per genus is followed, in parentheses, by number of species sampled here. The basis for associating each genus with Isoglossinae follows geographic range. For this table, we tabulate species in all genera that have been synonymized into *Stenostephanus* with that genus, although all new combinations have not yet been made. Genera treated by Lindau (1895) as Isoglossinae but since excluded and not listed here are *Herpetacanthus* (McDade & al., 2000a) and *Chlamydacanthus* (Manktelow & al., 2001).

Genus	No. of Species (Sampled Here)	Range, Basis for putative placement with Isoglossinae
Old World		
<i>Brachystephanus</i> Nees (including <i>Oreacanthus</i> Benth.; see Champluvier in prep.)	24 (7)	Tropical Africa, Madagascar; Lindau (1895), McDade & al. (2000a)
<i>Conocalyx</i> Benoist	1 (1)	Madagascar; androecium and pollen characters
<i>Forcipella</i> Baill.	5 (1)	Madagascar; pollen characters
<i>Isoglossa</i> Oerst. (including <i>Strophacanthus</i> Lindau)	50 (5)	Widespread in Africa, Arabia, Madagascar; Lindau (1895), McDade & al. (2000a)
<i>Populina</i> Baill.	2 (1)	Madagascar; Lindau (1895)
<i>Ptyssiglottis</i> T. Anderson	33 (2)	Southeast Asia to Papuaia; Hansen (1992), McDade & al. (2000a)
New World		
<i>Kalbreyeriella</i> Lindau	3 (1)	Lower Central America, Colombia; Leonard (1953), androecium and pollen characters
<i>Razisea</i> Oerst.	5 (2)	Mexico, Central America, Colombia; McDade & al. (2000a)
<i>Sphacanthus</i> Benoist	2 (0)	Madagascar; androecium and pollen characters

Stenostephanus Nees (including 82 (7) Mexico to Bolivia; Lindau (1895), Leonard (1953)
Cylindrosolenium Lindau,
Habracanthus Nees, *Hansteinia*
Oerst. *Kalbreyeracanthus* Wassh.,
Syringidium Lindau)

Table 2. Hypotheses of clades and of relationships among them. As indicated, hypotheses 2, 4, 5, and 7 are supported by at least some of the MP (maximum parsimony) and post burn-in Bayesian trees. Hypotheses 1, 3, 6, and 8 were contrasted with the MP result using the parsimony based Templeton test (z value) and likelihood based Kishino-Haswegawa RELL test (K-H RELL). For parsimony, we report the difference between the shortest trees and those consistent with the constraint (percent difference is calculated relative to lengths of the MP trees). For likelihood, we report the difference in likelihood scores between the unconstrained and constrained analyses.

Hypotheses	Results of Test
1. <i>Populina</i> is part of Isoglossinae	Reject: +22 steps, 2.2%, $z = -3.7730$, $P = 0.0002$; Diff -ln L = 109.264, K-H RELL, $P < 0.05$
2. <i>Ptyssiglottis</i> is part of Isoglossinae	Accept: Present in some MP and Bayesian trees
3. Old World Isoglossinae are monophyletic	Reject: +51 steps, 5.2%, $z = -6.5299$, $P < 0.0001$; Diff -ln L = 186.278, K-H RELL, $P < 0.05$
4. New World Isoglossinae are monophyletic	Accept: Present in some MP and Bayesian trees
5. Isoglossinae with monothealous stamens (i.e., <i>Brachystephanus</i> (including <i>Oreacanthus</i>), NW Isoglossinae) comprise a clade	Accept: Present in all MP and Bayesian trees
6. Isoglossinae with dithealous stamens (i.e., <i>Isoglossa</i> , <i>Conocalyx</i> , <i>Ptyssiglottis</i>) comprise a clade	Reject: +34 steps, 3.5%, $z = -5.0381$, $P < 0.0001$; Diff -ln L = 111.807, K-H RELL $P < 0.05$
7. <i>Isoglossa</i> (including <i>Conocalyx</i>) is monophyletic	Accept: Present in all MP and Bayesian trees
8. <i>Brachystephanus</i> (including <i>Oreacanthus</i>) is monophyletic	Cannot reject: +5 steps, 0.5%, $z = -1.3868$, $P = 0.1656$; Diff -ln L = 16.094, K-H RELL $P = 0.155$

Table 3. Characteristics of three DNA regions used here. (a) Aligned length includes all taxa sequenced for each locus. Data on variability, (b), (c) and (e), are reported for all taxa sequenced and for the *Brachystephanus* + NW Isoglossinae clade separately; (h) Missing data are reported as number of total of 26 in-group taxa and (i) as percent of sites (including only taxa for which any sequence data was available for that locus). Statistics for the *nrITS* region include 25 and 28 bp of the 18S and 26S ribosomal genes, respectively, that flank ITS1 and ITS2, plus the 5.8S gene.

Characteristic	<i>trnS/G</i> spacer	<i>trnT/L</i> spacer	<i>nrITS</i>
a. Aligned length	865	791	763
b. Variable sites (%): All taxa	186 (21.5%)	130 (16.4%)	220 (28.8%)
<i>Brachystephanus</i> + NW Isoglossinae only	59 (6.8%)	49 (6.2%)	62 (8.1%)
c. Parsimony informative sites (%): All taxa	91 (10.5%)	56 (7.1%)	99 (13.0%)
<i>Brachystephanus</i> + NW Isoglossinae only	16 (1.8%)	13 (1.6%)	13 (1.7%)
d. Parsimony informative indels	13	3	0
e. Pairwise distances, range: All Taxa	0.2 - 15.1%	0.3 - 9.1%	0.2 - 16.6%
<i>Brachystephanus</i> + NW Isoglossinae only	0.2 - 4.3%	0.2 - 3.0%	0.2 - 5.7%
f. Consistency index	0.841	0.877	0.786
g. Retention index	0.873	0.893	0.703
h. Missing data: # of taxa (of 26 in-group taxa)	1	1	5
i. Missing data (not including taxa for which entire sequence is missing)	10.6%	5.2%	8.2%

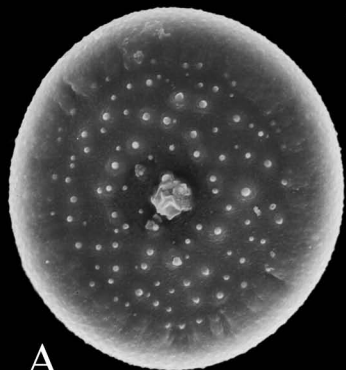
FIGURE LEGENDS

Figure 1. Pollen of Isoglossinae. A. *Stenostephanus chiapensis* (Breedlove 34374), apertural view. B. *Stenostephanus chiapensis* (Breedlove 34374), interapertural view. C. *Stenostephanus gracilis* (Breedlove 71524), apertural view. D. *Stenostephanus gracilis* (Breedlove 71524), interapertural view. E. *Ptyssiglottis nigrescens* (Burt 8312) from Hansen (1992), apertural view. F. *Ptyssiglottis auriculata* (Winkler 2745) from Hansen (1992), apertural view. Scales = 10 μm .

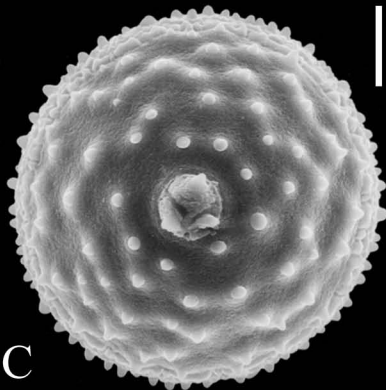
Figure 2. A. Opened corolla and bithecos stamens of Asian *Ptyssiglottis kunthiana* corollas are white with a yellow patch in the throat (redrawn from Fig. 20 in Hansen, 1992). B. Corolla and bithecos stamens of South African *Isoglossa ciliata*, corollas are white with pale purple nectar guides (drawn from liquid preserved material of *Balkwill et al. 11653* (PH). C. Flower of African *Brachystephanus myrmecophilus*, corollas are lilac to rose-colored (redrawn from Fig. 1 in Champluvier, 1994). D. Flower of *Stenostephanus tenellus*, corollas are pale lilac (drawn from a photograph taken by T. F. Daniel and Fig. 39H in Wasshausen & Wood, 2004). E. Flower of *Stenostephanus haematodes*, corollas are red to purplish red (redrawn from Fig. 11 in Daniel, 1999). F. Flower and monotheous anther of Central American *Razisea spicata*, corollas are bright red (drawn from a photograph taken by L. A. McDade).

Figure 3. Maximum a posterior (MAP) tree from Bayesian analysis of combined data set. Numbers above nodes are Bayesian posterior probabilities, below nodes are bootstrap values / decay indices. Names in parentheses indicate previous generic placement. *Stenostephanus silvaticus* is tentatively placed because data were available only from *nrITS*. *Forcipella* is not shown because it was placed outside of Justiciaeae. Hypotheses regarding the evolution of Gürtelpollen and monotheous stamens are indicated with arrows. * Champluvier (in prep.) synonymizes *Oreacanthus* with *Brachystephanus* and describes a number of new taxa of *Brachystephanus*, two of which are included here.

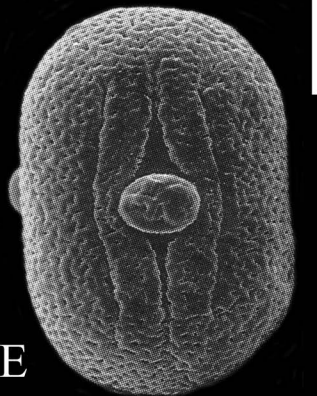
Figure 4. One randomly chosen MP tree (of 267 trees, length = 981, CI = 0.837, RI = 0.837); branch lengths proportional to number of changes as optimized by ACCTRAN in PAUP. * Champluvier (in prep.) synonymizes *Oreacanthus* with *Brachystephanus* and describes a number of new taxa of *Brachystephanus*, two of which are included here. Note very short branches among *Brachystephanus* and NW Isoglossinae; only *Oreacanthus manni* + *B.* sp. nov. 1 and the two species of *Razisea* are resolved with strong support in the MAP tree (see Fig. 3).



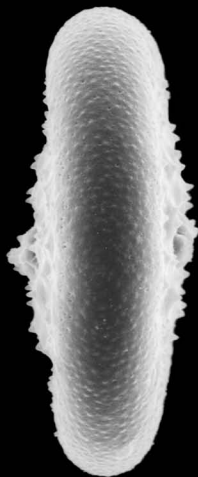
A



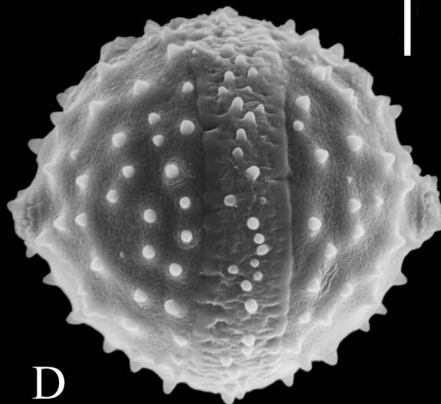
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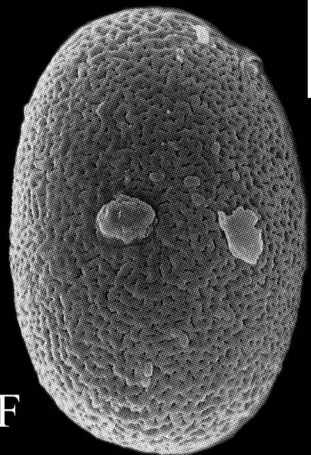
E



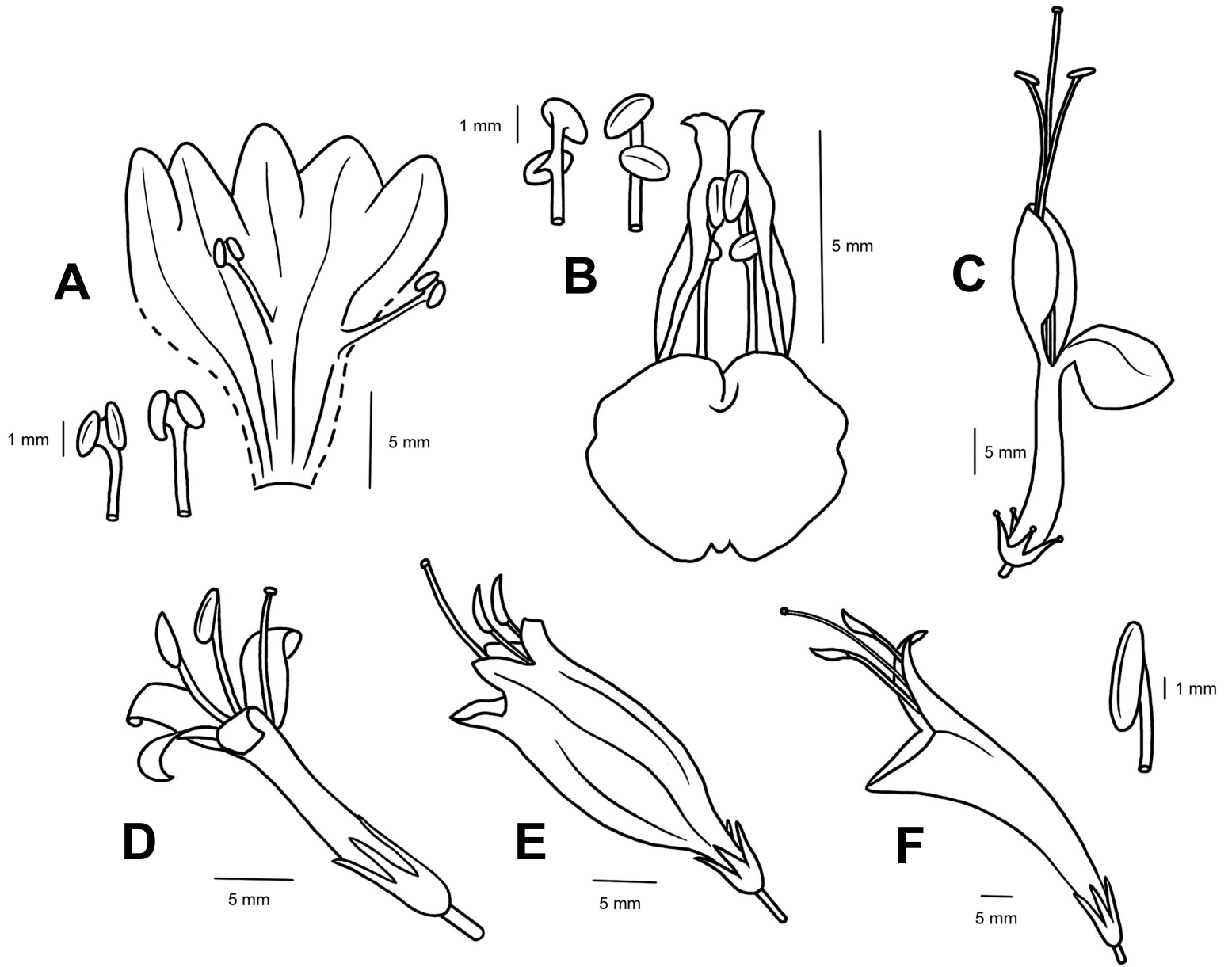
B

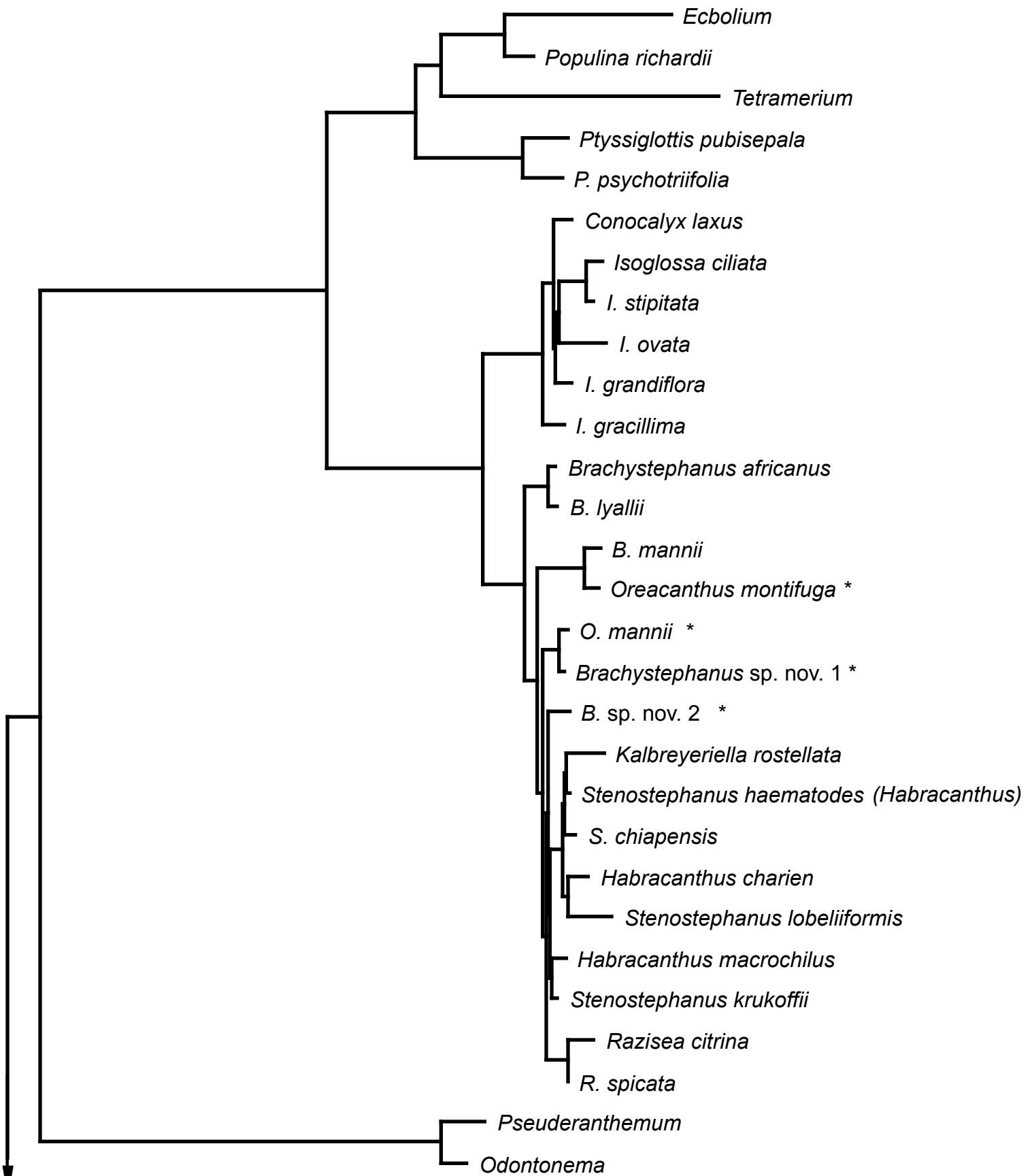


D



F





Ecboium

Populina richardii

Tetramerium

Ptyssiglottis pubisepala

P. psychotriifolia

Conocalyx laxus

Isoglossa ciliata

I. stipitata

I. ovata

I. grandiflora

I. gracillima

Brachystephanus africanus

B. lyallii

B. mannii

Oreacanthus montifuga *

O. mannii *

Brachystephanus sp. nov. 1 *

B. sp. nov. 2 *

Kalbreyeriella rostellata

Stenostephanus haematodes (Habracanthus)

S. chiapensis

Habracanthus charien

Stenostephanus lobeliiformis

Habracanthus macrochilus

Stenostephanus krukoffii

Razisea citrina

R. spicata

Pseuderanthemum

Odontonema

Out-groups

— 10 changes

