# Molecular Phylogenetics of Phyllanthaceae: Evidence from Plastid MatK and Nuclear PHYC Sequences

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# MOLECULAR PHYLOGENETICS OF PHYLLANTHACEAE: EVIDENCE FROM PLASTID *MATK* AND NUCLEAR *PHYC* SEQUENCES[1]

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Plastid *matK* and a fragment of the low-copy nuclear gene *PHYC* were sequenced for 30 genera of Phyllanthaceae to evaluate tribal and generic delimitation. Resolution and bootstrap percentages obtained with *matK* are higher than that of *PHYC*, but both regions show nearly identical phylogenetic patterns. Phylogenetic relationships inferred from the independent and combined data are congruent and differ from previous, morphology-based classifications but are highly concordant with those of the plastid gene *rbcL* previously published. Phyllanthaceae is monophyletic and gives rise to two well-resolved clades (T and F) that could be recognized as subfamilies. DNA sequence data for *Keayodendron* and *Zimmermanniopsis* are presented for the first time. *Keayodendron* is misplaced in tribe Phyllantheae and belongs to the *Bridelia* alliance. *Zimmermanniopsis* is sister to *Zimmermannia*. *Phyllanthus* and *Cleistanthus* are paraphyletic. *Savia* and *Phyllanthus* subgenus *Kirganelia* are not monophyletic.

**Key words:** Malpighiales; *matK*; molecular phylogenetics; Phyllanthaceae; *PHYC*; systematics.

Phyllanthaceae are a morphologically diverse pantropical family of about 2000 species in c. 60 genera. They have been segregated along with Pandaceae, Picrodendraceae, and Putranjivaceae from Euphorbiaceae sensu lato (s.l.), following recent molecular work (Savolainen et al., 2000; APG II, 2003). The molecular systematics of Phyllanthaceae have been investigated as part of a larger multigene study on the systematics of Euphorbiaceae s.l. The largest sampling used plastid *rbcL* sequences, and over 350 Euphorbiaceae s.l. sequences including 76 (74 taxa) of Phyllanthaceae, to assess subfamilial and tribal relationships (i.e., Wurdack and Chase, 1999; Wurdack, 2002; Wurdack et al., in press). Two clades of Phyllanthaceae found in these molecular analyses (Wurdack et al., in press, and here) are nearly identical to the suprageneric classification of Webster (1994) and Radcliffe-Smith (2001), but the remaining clades do not correspond to previous tribal classifications. For a more detailed history of Phyllanthaceae classification, see Wurdack et al. (in press).

The *matK* gene is one of the most rapidly evolving plastid protein-coding regions (Wolfe, 1991). It is approximately 1550 base pairs (bp) long and encodes a maturase involved in splicing type II introns from RNA transcripts (Wolfe et al., 1992). Recent studies have shown the usefulness of this gene for resolving intergeneric or interspecific relationships among flowering plants, e.g., Malpighiaceae (Cameron et al., 2001), Poaceae (Liang and Hilu, 1996), Cornaceae (Xiang et al., 1998), *Nicotiana* (Aoki and Ito, 2000; Clarkson et al., in press), *Chrysosplenium* (Soltis et al., 2001), *Hypochaeris* (Samuel et al., 2003), Orchidaceae (Goldman et al., 2001; Salazar et al., 2003) and most recently across all angiosperms (Hilu et al., 2003).

Low-copy nuclear protein-coding genes remain underutilized in phylogenetic studies, despite the need for nuclear comparisons with trees produced from plastid regions (Doyle, 1992, 1997). The nuclear regions most commonly used in phylogenetic studies are from high-copy ribosomal loci, such as ITS (Baldwin et al., 1995). The multigene phytochrome (*PHY*) family is a potential source of phylogenetic information. Phytochromes are photoreceptors for red and far-red light in all land plants (Quail, 1991) and mediate diverse developmental responses throughout the life cycle of a plant. In angiosperms, five related sequences coding for phytochrome proteins designated *PHYA-PHYE* have been characterized in *Arabidopsis thaliana* (Sharrock and Quail, 1989; Clack et al., 1994). A simple way to sample putatively orthologous loci in the phytochrome gene family is to use locus-specific amplification primers. Phytochrome sequence data have provided a high degree of resolution within basal angiosperms (Mathews and Donoghue, 1999), Fabaceae (Mathews et al., 1995), Poaceae–Andropogoneae (Mathews et al., 2002), Malpighiaceae (Davis et al., 2002), and Malpighiales (Davis and Chase, 2004) and may be useful for resolving relationships within Phyllanthaceae. The overall rate of evolution of the *PHY* lineage is about 10 times faster than *rbcL* (Mathews et al., 1995).

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TABLE 1. Primer sequences used in this study for the amplification and sequencing of the *trnK* intron and *matK* gene.

| Primer | Sequence |
|---|---|
| *trnK* 570F | 5′-TCC AAA ATC AAA AGA GCG ATT GG-3′ |
| 80F | 5′-CTA TAC CCA CTT ATC TTT CGG GAG T-3′ |
| 390F | 5′-CGA TCT ATT CAT TCA ATA TTT C-3′ |
| 800F | 5′-CAT GCA TTA TGT TAG ATA TCA AGG-3′ |
| 1200F | 5′-GA (CT) TCT GAT ATT ATC AAC CGA TTT G-3′ |
| 190R | 5′-ATT CGA GTA ATT AAA CGT TTT ACA A-3′ |
| 530R | 5′-GTT CCA ATT CCA ATA CTC GTG AAG-3′ |
| 950R | 5′-AAA AT(AG) ACA TTG ACA TAA ATT GAC AA (AG) G-3′ |
| 1300R | 5′-CGA AGT ATA TA (CT) TT (CT) ATT CGA TAC A-3′ |
| 1710R | 5′-GCT TGC ATT TTT CAT TGC ACA CG-3′ |

This study analyzes the nuclear gene *PHYC* and the plastid gene *matK* to infer phylogenetic relationships within Phyllanthaceae and determine congruence of these two regions. We aim furthermore to evaluate the phylogenetic patterns obtained with *rbcL* sequence data (Wurdack et al., in press) with additional genetic markers as the basis for creating a revised tribal classification of Phyllanthaceae.

## MATERIALS AND METHODS

***Plant material***—Forty-seven species (49 different accessions) from 30 genera (of the 60 genera recognized by Radcliffe-Smith [2001]) for Euphorbiaceae-Phyllanthoideae, representing five of 10 tribes, and six of 11 subtribes of Antidesmeae and Phyllantheae were included in the analyses (see Supplemental Data accompanying the online version of this article http://ajbsupp.botany.org/ßß). The taxon set used in the *matK* analysis included 41 ingroup species (43 accessions) and excluded *Keayodendron*, whereas the analysis of *PHYC* included 44 ingroup species (45 accessions) and excluded *Uapaca*. Outgroup taxa for these analyses included representatives from several other families of Malpighiales (APG II, 2003) including: Clusiaceae, Euphorbiaceae sensu stricto (s.s.), Humiriaceae, Ochnaceae, Picrodendraceae, and Putranjivaceae (see Appendix 1 in supplemental data accompanying the online version of this article http://ajbsupp.botany.org/ßß). Forty-one species (42 accessions) representing all 30 sampled genera were analyzed in combination. Because of our focus on Phyllanthaceae, and due to the limited sampling of other Malpighialean lineages, no close relationship among outgroup families should be inferred from our results. Most samples were obtained from the DNA Bank of the Royal Botanic Gardens, Kew, UK (http://www.rbgkew.org.uk/data/dnaBank/homepage.html). In addition, silica gel dried specimens collected in Madagascar and Sri Lanka were included.

***DNA extraction and amplification***—Total DNA was extracted from material stored in silica gel following the 2 × CTAB (cetyltrimethyl ammonium bromide) procedure of Doyle and Doyle (1987). Most of the DNA samples obtained from herbarium specimens were purified by cesium chloride/ethidium bromide gradient (1.55 g/mL). Polymerase chain reaction (PCR) amplification was carried out using PCR ready mix (AB-0619/LD from Abgene, Vienna, Austria) and 2–8 ng (1 μL of 2–8 ng/μL) of template total DNA for a 50 μL reaction mixture. The PCR profile consisted of an initial 2-min premelt at 94°C and 35 cycles of 1-min denaturation at 94°C, 30-s annealing at 48°C, and 1-min extension at 72°C followed by a final extension of 10-min at 72°C. Amplified fragments were checked with 1% agarose gel, and the double-stranded DNA fragments were purified using QIAquick gel purification kit (Qiagen, Margaritella, Vienna, Austria).

We designed new amplification primers for *matK* spanning the entire region plus part of the *trnK* intron 5′(*trnK* 570F) and *trnK*3′ (1710R) (Table 1). Figure 1 shows the positions of the *trnK* intron in which *matK* is embedded and the positions of the primers used in this study. Degraded DNA from herbarium specimens was amplified in 5 or 6 fragments that were sequenced separately and then combined into a single contig.

For the *PHYC* gene, we designed our own primers from the available sequences in GenBank. Initially PCR products were cloned by ligation into pGEM-T Vector Systems (Promega Gmbh, Mannheim, Germany); XL1-Blue competent cells were transformed according to the manufacturer's protocol (Stratagene Europe, Amsterdam, The Netherlands). Resulting colonies were screened for plasmids with inserts, and five positive clones were amplified and then sequenced using the same primers. To avoid time-consuming cloning, another set of primers from the aligned cloned sequences of some species was designed *PHYC*-F [5′-CCAGCTACTGATATACCTCAAGCTTC-3′] and *PHYC*-R [5′-CCAGCTTCCATAAGGCTATCAGTACT-3′], which enabled us to directly sequence a fragment of approximately 600 bp. This fragment is in the first exon of the *PHYC* gene starting from 800 bp downstream. The entire gene is 3571 bp long in *Arabidopsis thaliana* and has two introns, 136 and 98 bp in length. Primer positions of the sequences used in this study are shown in Fig. 2.

***Sequencing***—The purified fragments were directly sequenced on an ABI 377 automated sequencer (Applied Biosystems, ABI, Vienna, Austria) using dye terminator chemistry following the manufacturer's protocol. Cycle sequencing reactions were performed for each template using each of the two primers used for PCR amplification and internal primers for *matK* if required. Both strands were sequenced. The programs Sequence Navigator and AutoAssembler (ABI) were used to edit and assemble the complementary sequences. These sequences have been deposited in GenBank (Appendix 1 in Supplemental Data that accompanies the online version of this article http://ajbsupp.botany.org/ßß).

***Sequence alignment and phylogenetic analyses***—Alignments were obtained using the program Clustal V (Higgins et al., 1992) and improved by visual refinement. In the *matK* sequences, large gaps (in multiples of three) were often needed. Individual and combined parsimony analyses of *matK* and

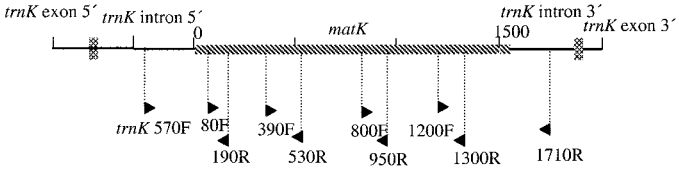


Fig. 1. Location of the *matK* gene in the *trnK* intron. Arrowheads indicate the location and direction of the primers. The *trnK* 3914F primer is from Johnson and Soltis (1994); all others are new to this study.

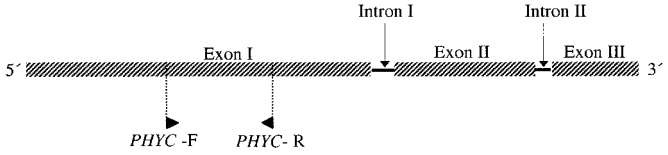


Fig. 2. *PHYC* (55716–59286) in the fifth chromosome of *Arabidopsis thaliana* (Genbank accession number AB005236). Arrowheads (*PHYC* F and *PHYC* R) indicate positions of our primers.

*PHYC* were performed using PAUP (version 4.0b10; Swofford, 2002). All heuristic searches were conducted with equal weights, 1000 replicates of random sequence addition, tree bisection-reconnection (TBR) branch swapping, and MulTrees on but permitting 10 trees to be held at each step to enable more replicates to be performed in less time. Indels were treated as missing data in our analyses. Confidence limits (BP, bootstrap percentages) for clades were assessed by performing 1000 replicates of bootstrapping (Felsenstein, 1985) using equal weighting, TBR swapping, MulTrees on, and holding 10 trees per replicate. The individual bootstrap consensus trees were inspected visually to determine congruence of the two data sets (Whitten et al., 2000).

Model evaluation was done for both matrices independently using Mr Bayes 2.01 (Huelsenbeck and Ronquist, 2001) to find the best fit. Because both genes had similar models an independent, model-based estimate was produced for the combined sequence data using Bayesian inference (Larget and Simon, 1999) with the method implemented in MrBayes. The general time reversible model (GTR + I + G, nst = 6, rate = invgamma) was chosen for sequence evolution (Rodríguez et al., 1990). Four Markov chains starting with a random tree were run simultaneously for one million generations, sampling trees at every 100th generation. Trees prior to stationarity (3000 trees) were excluded and the remaining trees used to construct in PAUP* a consensus tree with percentages (Bayesian posterior probabilities [PP]) of trees compatible with the single tree. We will not report posterior probabilities here because these have been demonstrated to be overestimates of confidence (Suzuki et al., 2002).

## RESULTS

***Analysis of the matK gene***—The aligned *matK* matrix consisted of 1612 bp, of which 840 positions were variable, and 602 (37%) were potentially parsimony informative. Heuristic searches generated three equally parsimonious trees with 1845 steps. One of the equally parsimonious trees with Fitch lengths (DELTRAN, delayed transformation, optimization) above each branch and bootstrap percentages (BP > 50) below each branch is shown in Fig. 3.

Phyllanthaceae are weakly supported (BP 60) as monophyletic and are split into two strongly supported (both BP 100) clades T and F (tanniniferous and fasciculate clade, respectively). The first major clade (T) corresponds to tribes Antidesmeae + Bischofieae sensu Radcliffe-Smith (2001) and contains four subclades. One strongly supported (BP 100) subclade within clade T includes *Aporosa* and *Baccaurea* (Antidesmeae-Scepinae); a second (BP 100) includes *Apodiscus* (Antidesmeae-Scepinae) as sister to the members of Antidesmeae-Antidesminae included in this analysis, *Antidesma* + *Thecacoris* (BP 100); the third and fourth *Uapaca* (monogeneric Antidesmeae-Uapacinae) and *Bischofia* (monotypic Bischofieae), respectively, are weakly placed relative to one another and to the other two subclades within clade T. Each of the two representative species of *Baccaurea* and *Thecacoris* are moderately well supported (BP 82 and 87, respectively) as sister taxa.

The second major clade (F) is split into four well-supported (BP 100) subclades (F1, F2, F3, F4). The first (F1) comprises all members of Phyllantheae-Flueggeinae sensu Webster (1994) included in this analysis. *Flueggea* is sister to the weakly supported (BP 60) clade containing the remaining members of clade F1. *Margaritaria* is then sister to a strongly supported (BP 100) clade containing all genera with phyllanthoid branching (Webster, 1956). *Phyllanthus* is not monophyletic and falls into two clades each with BP 100. The first contains *Phyllanthus calycinus* (subgenus *Isocladus*) and *P.* cf. *fuscoluridus* + *P.* cf. *mantsakariva* (both subgenus *Kirganelia* section *Anisonema*, supported as sisters with BP 100). In the second, *Phyllanthus nummulariifolius* (subgenus *Kirganelia* section *Pentandra* sensu Webster [1967] or subgenus *Tenellanthus* nomen invalidum sensu Brunel [1987]) is sister to the well-supported (BP 99) clade comprising *Glochidion* plus (*Breynia* + *Sauropus*). The latter two genera are strongly supported as sisters with BP 100. The two species of *Breynia* and *Flueggea* were each identified as monophyletic with BP 100 and 94, respectively.

Subclades F2, F3, and F4 are united in a weakly supported clade (BP 52). The second subclade (F2) is well supported (BP 100) and consists of *Bridelia*, *Cleistanthus* (both tribe Bridelieae), *Pseudolachnostylis* (Phyllantheae-Pseudolachnostylidinae), *Gonatogyne*, *Lachnostylis*, and *Savia* pro parte (all Wielandieae). *Lachnostylis* is sister to a clade (BP 100) of the remaining members of F2, which are split into two subclades; one with *Gonatogyne* + *Savia dictyocarpa* (BP 100), and the other with *Bridelia*, *Cleistanthus* and *Pseudolachnostylis* (BP 99). *Cleistanthus* is not monophyletic. *Cleistanthus oblongifolius* is more closely related to *Bridelia* (BP 100) than it is to *Cleistanthus perrieri*.

The third strongly supported (BP 100) subclade (F3) includes *Actephila* (Wielandieae), *Leptopus* (Phyllantheae-Leptopinae), *Meineckia*, *Zimmermannia*, *Zimmermanniopsis* (Phyllantheae-Pseudolachnostylidinae), and *Poranthera* (Antidesmeae-Porantherinae). *Poranthera* is strongly supported (BP 100) as sister to a well-supported clade (BP 97) clade constituting the remaining members of clade F3. Within this clade, *Actephila* + *Leptopus* form a strongly supported (BP 100) subclade. The two sampled species of *Leptopus* (both Old World species) are also supported by BP 100. The other subclade (BP 100) contains *Meineckia* + *Zimmermannia* + *Zimmermanniopsis*, with *Meineckia* sister to the other two taxa (BP 86). *Heywoodia* (Wielandieae) is weakly supported (BP 52) as sister to subclade F3.

The fourth well-supported subclade (F4; BP 100) includes all lineages of the western Indian Ocean Wielandieae. *Wielandia* is weakly supported as sister to all other species in this subclade (BP 57).

***Analysis of the PHYC gene***—The aligned *PHYC* matrix consisted of 601bp of which 485 were variable and 391 (65%) were potentially parsimony informative. Heuristic searches on this data set resulted in 1816 equally most parsimonious trees with 1861 steps. One of the equally parsimonious trees with Fitch lengths (DELTRAN optimization) above each branch and bootstrap percentages (BP > 50) below each branch is shown in Fig. 4.

The monophyly of Phyllanthaceae is weakly supported (BP < 50), and the two major clades, T and F, found in *matK* were recovered with weak support (BP 67 and 54, respectively; see Fig. 4). The composition of clades T and F1–F4 is identical to those uncovered using *matK* (with the omission of *Uapaca* from clade T, and the addition of *Keayodendron* to clade F2 due to sampling differences). The topology of subclade T was identical between *PHYC* and *matK* for all similarly sampled taxa, but there are some differences in the placement of individual taxa in subclades F1–F4.

Clade F1 is moderately supported (BP 82). In contrast to the *matK* analysis, *Margaritaria* is sister to the remaining members of clade F1. *Flueggea* is monophyletic (BP 100), and is well-supported (BP 90) as sister to the clade (BP 94) characterized by phyllanthoid branching (Webster, 1956). *Phyllanthus* is not monophyletic; species of *Phyllanthus* occur in at

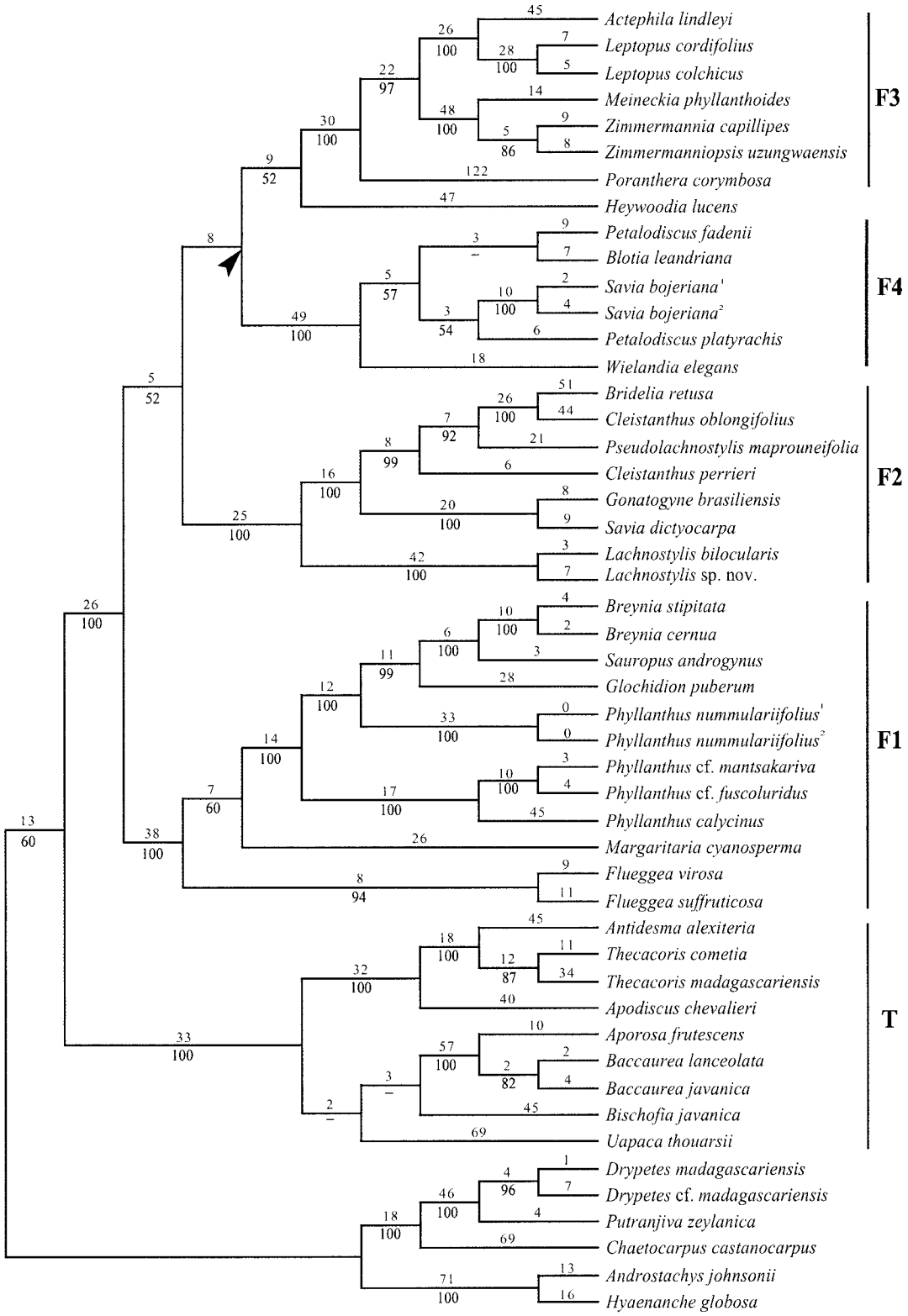


Fig. 3. One of the three most parsimonious trees obtained from the maximum parsimony analysis of the *matK* gene (length = 1845, CI = 0.63, RI = 0.82). Branch lengths (DELTRAN optimization) and bootstrap percentages (>50) are indicated above and below the branches, respectively. Arrowheads indicate nodes not present in the strict consensus tree. Hyphens (-) indicate BP < 50.

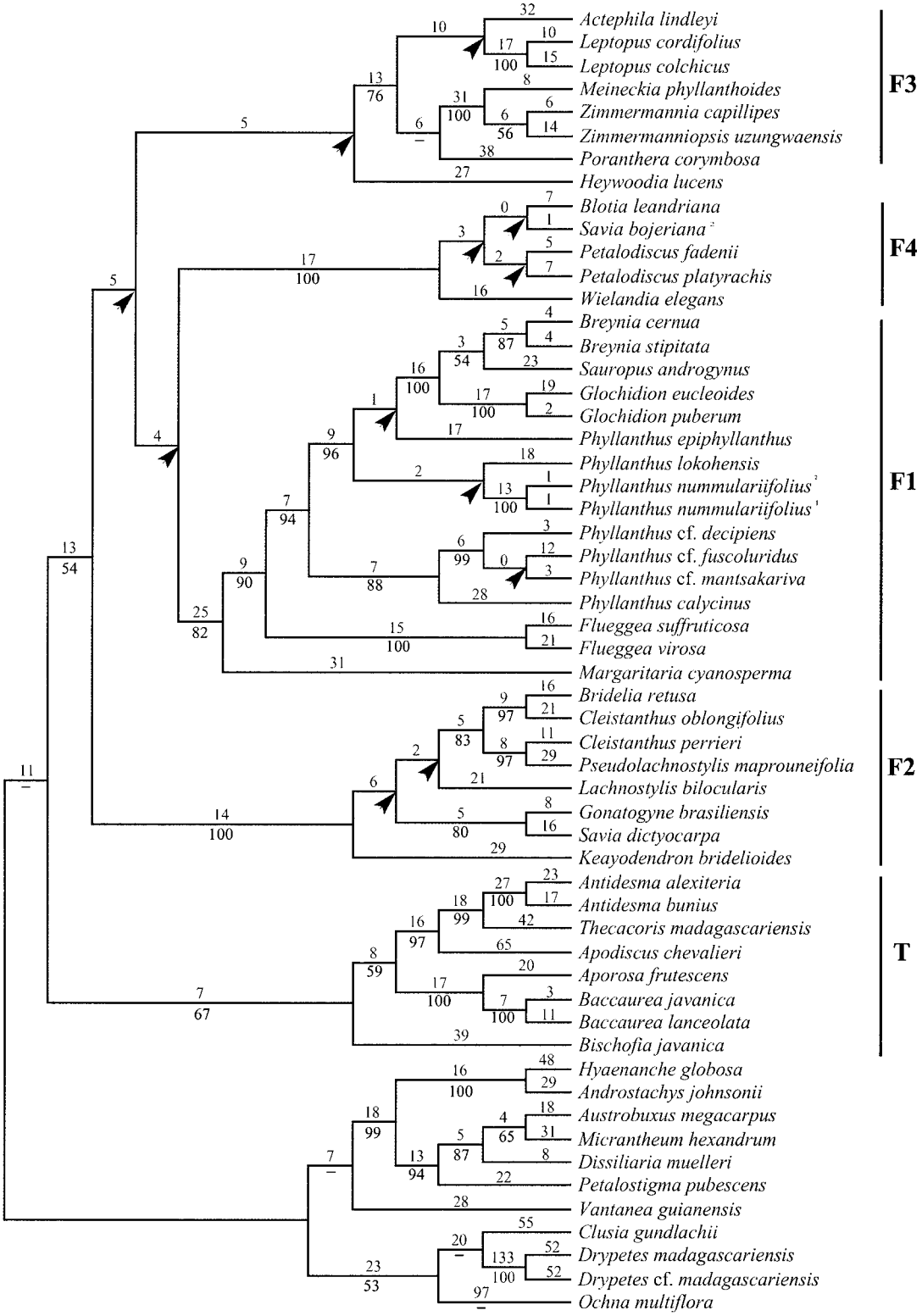


Fig. 4. One of the most parsimonious trees obtained from the maximum parsimony analysis of the *PHYC* (length = 1861 steps, CI = 0.45, RI = 0.65). Branch lengths (DELTRAN optimization) and bootstrap percentages (>50) are indicated above and below the branches, respectively. Arrowheads indicate nodes not present in the strict consensus tree. Hyphens (-) indicate BP < 50.

least two clades. The first (BP 88) includes *P. calycinus* (subgenus *Isocladus*), plus a well-supported (BP 99) clade consisting of three taxa of subgenus *Kirganelia* section *Anisonema*. The second clade (BP 96) is a polytomy comprised of several species of *Phyllanthus* [*P. lokohensis* (subgenus *Phyllanthus*), plus two accessions of *P. nummulariifolius* (*Kirganelia-Pentandra* or *Tenellanthus*) and *P. epiphyllanthus* (subgenus *Xylophylla*)], plus the well-supported (BP100) clade *Glochidion* (*Breynia* + *Sauropus*). Within the last subclade, *Sauropus* + *Breynia* are weakly supported as sisters (BP 54), and support for the two species of *Breynia* is moderate (BP 87).

*Bridelia* and *Cleistanthus* (both Bridelieae), *Keayodendron* and *Pseudolachnostylis* (both Phyllantheae-Pseudolachnostylidinae), *Gonatogyne*, *Savia* and *Lachnostylis* (all Wielandieae) form a strongly supported (BP 100) clade F2. As with *matK*, *Gonatogyne* + *Savia* on the one hand, and *Bridelia*, *Cleistanthus* and *Pseudolachnostylis* on the other hand form well-supported (BP 80 and 83) clades. *Cleistanthus* appears non monophyletic as in *matK*. *Cleistanthus oblongifolius* clusters with *Bridelia* again (BP 97), but *C. perrieri* forms a well-supported (BP 97) clade with *Pseudolachnostylis*. *Keayodendron*, which was not sampled in the *matK* analysis, is weakly supported as sister to all other members of clade F2.

Clade F3 is weakly supported (BP 76) and consists of two sister clades: one containing *Actephila* and *Leptopus*, and the other *Poranthera* and *Meineckia* (*Zimmermannia* + *Zimmermanniopsis*). Each of these clades is supported with BP $< 50$. The two species of *Leptopus* are united by BP 100. *Meineckia* (*Zimmermannia* + *Zimmermanniopsis*) is supported by BP 100, and the sister-group relationship of *Zimmermannia* and *Zimmermanniopsis* has weak support (BP 56). The position of *Heywoodia* is weakly supported as sister to F3, a placement identical to that inferred from *matK*.

The western Indian Ocean Wielandieae again group in the highly supported (BP 100) clade F4. No further bootstrap supported resolution is obtained with *PHYC* in this clade.

***Parsimony and Bayesian analysis of combined data***—Since the consensus trees obtained with the individual gene matrices were topologically congruent, the two data sets were combined for further analysis. The aligned combined *matK* and *PHYC* matrix consisted of 2277bp. The heuristic search on this data set resulted in six equally most parsimonious trees with 3440 steps (Fig. 5). Bayesian analyses of the combined matrix produced a tree (not shown) that is nearly identical to the parsimony tree. All clades with high posterior probabilities (PP 1.0) are also present and receive at least moderate bootstrap support in the parsimony analysis.

The most notable result of this combined parsimony analysis is the high support (BP 100) for Phyllanthaceae. The two major clades (T and F) are well-supported (both with BP 100). The topology of clade T is identical with that in the *matK* tree, with similarly high bootstrap percentages. Clades F1–F4 are all well supported (BP 100) and resolved into F1, F2, and (F4 (*Heywoodia* + F3)).

Clade F1 is strongly supported (BP 100). The positions of *Flueggea* and *Margaritaria* equal those in the *PHYC* analysis. The topology of all other nodes agrees with both single-gene analyses, but bootstrap percentages vary slightly.

Clade F2 is well-supported in the combined analysis (BP 100). The topology of the strict consensus tree is identical to that of the *PHYC* analysis, having low support for internal nodes. Clade F3 shows an identical topology and similarly high bootstrap percentages in the combined and *matK* analyses. Placement of *Heywoodia* as sister of the F3 clade is moderately supported (BP 86), compared to the weak support in the single-region analyses. Clade F4 is supported by BP 100 in all three analyses, but support for internal nodes does not increase in the combined analysis as in clade F2.

## DISCUSSION

***matK and PHYC***—The utility of *matK* for resolving generic or species level relationships is similar or greater than that of nuclear rDNA ITS (Soltis et al., 1996). Indels are likely to be present in a *matK* data matrix of any taxonomic breadth. In our analysis *matK* resolves clades well at the tribal and generic levels and provides high bootstrap percentages for the different clades (Fig. 3). Although there was a greater percentage of potentially informative sites in *PHYC* (65%) than in *matK* (37%), the latter gene provided higher bootstrap percentages for the major clades and appears to be of greater phylogenetic utility.

***Comparison with rbcL***—For ease of reference, the clades recovered in this study are named in concordance with the *rbcL* analysis of Wurdack et al. (in press). Sampling for the *rbcL* study (Wurdack et al., in press) was more comprehensive than in this study. All genera included here were also included in the *rbcL* study, with the exception of *Keayodendron* (clade F2) and *Zimmermanniopsis* (clade F3). This study is also the first to include representatives of *Phyllanthus* subgenus *Kirganelia* section *Anisonema* (clade F1). The topologies of the combined *matK*/*PHYC* (presented here) and *rbcL* trees are consistent with one another. Major clades of Phyllanthaceae (T, F1–F4) recovered with *rbcL* were also found with *matK* and *PHYC*. A single inconsistency in the topologies of the three genes is the placement of *Cleistanthus perrieri* (clade F2). The position of this species in the *rbcL* tree is identical to that in the *matK* tree (sister to *Pseudolachnostylis* (*Bridelia* + *Cleistanthus oblongifolius*)) but differs from the *PHYC* tree and the combined *matK*/*PHYC* tree (sister to *Pseudolachnostylis* only). It is possible that there is conflicting signal for the position of *Pseudolachnostylis*. Paraphyly of *Cleistanthus* was already reported and discussed in Wurdack et al. (in press).

The combined *matK*/*PHYC* tree shows higher support for individual clades and better resolution than that obtained from *rbcL*. It should be noted that outgroup sampling in these studies is not identical and may affect support for the Phyllanthaceae node. Most prominently, monophyly of Phyllanthaceae is supported with BP 100 (rather than just BP 73 with *rbcL*). The two major clades (T and F) have slightly improved support (BP 100 for both vs. BP 98 and 91 in *rbcL*). Support for the subclades F1–F4 has increased from BP 95–100 to BP 100 for all four clades in the combined analysis.

Monophyly of *Thecacoris* is confirmed in the *matK* and combined analysis with moderate support (BP 87 and 86, respectively). The sampled species represent the two major groups, *Thecacoris* s.s. and *Cyathogyne*, recognized at generic rank by some authors (e.g., Pax and Hoffmann, 1922; Léonard, 1995). Their relationship received BP $< 50$ in the *rbcL* analysis.

Two more instances of improved resolution are noted here with the caveat that sampling in the *rbcL* analysis was more comprehensive in these clades: *Antidesma* + *Thecacoris* are

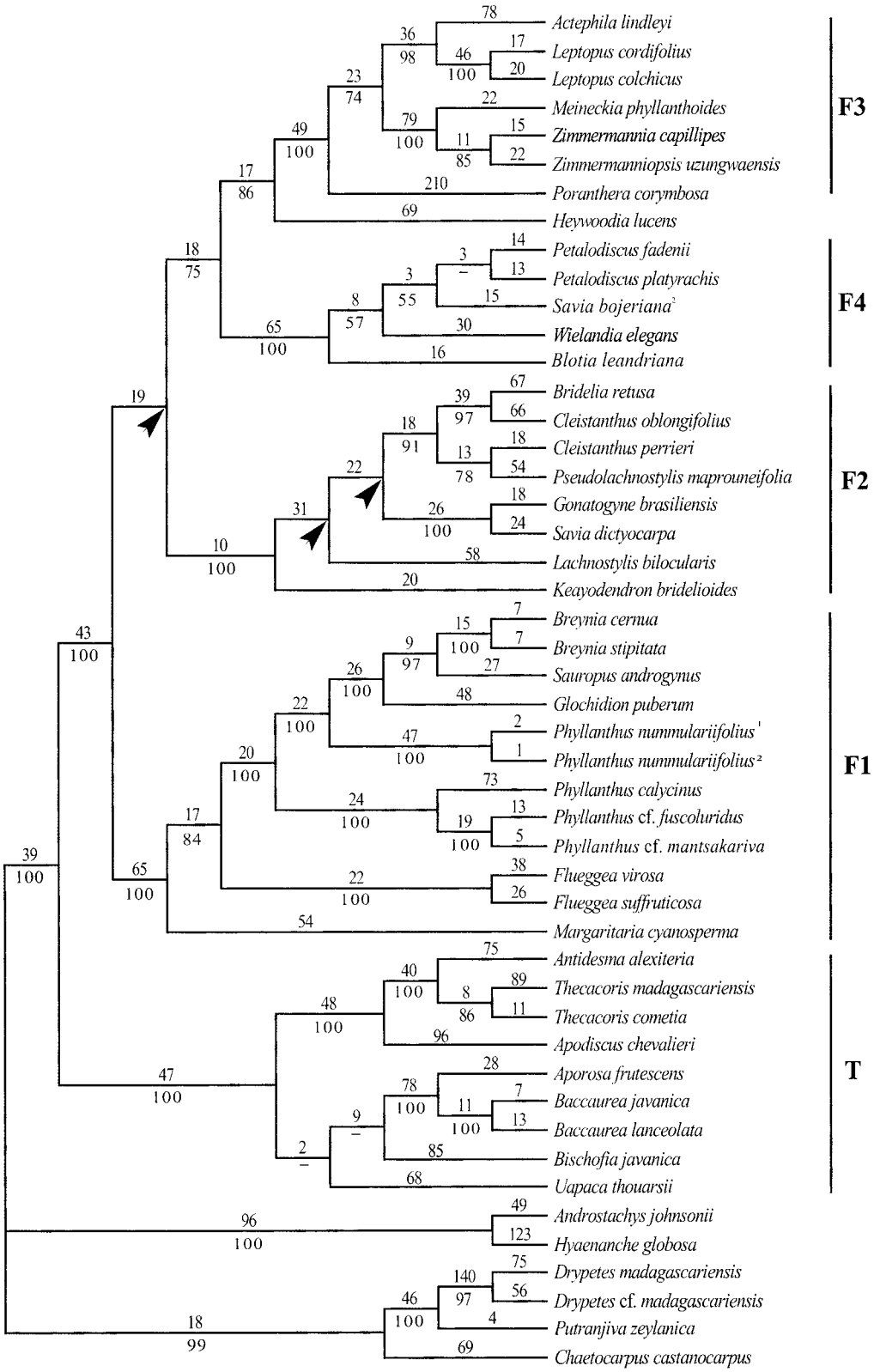


Fig. 5. One of the six most parsimonious trees of the combined analysis of *matK* and *PHYC* (length = 3440, CI = 0.59, RI = 0.75). Branch lengths (DELTRAN optimization) and bootstrap percentages (>50) are indicated above and below the branches, respectively. Arrowheads indicate nodes not present in the strict consensus tree. Hyphens (-) indicate BP < 50.

strongly supported sister taxa with *Apodiscus* sister to both, whereas with *rbcL* no further resolution was obtained for these three taxa. *Antidesma* and *Thecacoris* closely resemble each other, and the genera lack distinguishing generic characters in staminate specimens (both are dioecious). In pistillate specimens, the unilocular drupes of *Antidesma* are clearly different from the trilocular schizocarps of *Thecacoris*. In the *matK* and combined analyses, *Leptopus* and *Actephila* are grouped together (BP 98), which is biogeographically plausible (both are distributed in Asia and Australia) even though it contradicts wood anatomical (Mennega, 1987) and embryological (Webster, 1994) arguments used to distance *Actephila* (previously in Wielandieae) from *Leptopus* (Phyllantheae-Leptopinae).

***Drypetes madagascariensis***—The high genetic divergence of the two accessions of *Drypetes madagascariensis* (Putranjivaceae, outgroup for this study) may indicate heterogeneity of the species in its present circumscription. Most species of the dioecious genus *Drypetes* have few distinguishing morphological characters, and *D. madagascariensis* is noted for its remarkable variability (McPherson, 2000). The accession here marked as *D.* cf. *madagascariensis* differs from the majority of specimens solely by the lack or poor development of the fifth sepal but agrees in all other macro-morphological characters (the specimen is in fruit) with *D. madagascariensis*.

***Position of Zimmermanniopsis***—*Zimmermanniopsis uzungwaensis* has been variously accepted at generic rank (Radcliffe-Smith and Harley, 1990; Webster, 1994; Radcliffe-Smith, 2001) or included in *Meineckia* as section *Zimmermanniopsis* (Radcliffe-Smith, 1997; Govaerts et al., 2000). Placement in all analyses presented here confirms the close relationship of *Zimmermanniopsis* to *Zimmermannia*, and to *Meineckia* but more sampling is needed to determine the status of these taxa. A more comprehensive study of subclade F3 is presently underway at the Royal Botanic Gardens, Kew. One objective of this study is to clarify the taxonomy of the *Meineckia*/*Zimmermannia*/*Zimmermanniopsis*-complex.

***Placement of Keayodendron***—The monotypic genus *Keayodendron* has not previously been included in molecular phylogenetic analyses. Initially described as a species of *Casearia* (formerly Flacourtiaceae; Salicaceae-Samydeae in Chase et al., 2002), Leandri (1959) transferred it correctly to Euphorbiaceae-Phyllanthoideae and described the new genus *Keayodendron*. He positioned it near *Drypetes* (now Putranjivaceae) because of the general resemblance of leaves and inflorescences, but also pointed out similarities to *Bridelia* in floral and embryo morphology, most strikingly the extrastaminal staminate disc in *Bridelia* and *Keayodendron* (vs. a central staminate disc in *Drypetes*). He furthermore compared his new genus to *Pseudolachnostylis* and *Securinega*. The resemblance between *Keayodendron* and *Bridelia* had already been noted in the basionym *Casearia bridelioides* Gilg ex Engl. However, emphasis placed on the valvate sepals in tribe Bridelieae previously obscured the close relationship of those taxa. Webster (1994: 41–42) placed *Keayodendron* in Phyllantheae-Pseudolachnostylidinae ''for lack of a better alternative,'' stating that ''. . . it is quite possible that *Pseudolachnostylis* and *Keayodendron* may not be closely related to the rest of the genera.'' He compared its fruits and aspect to *Bridelia*, but the lack of petals and the imbricate sepals in *Keayodendron* deterred him from formally associating it with Bridelieae. Radcliffe-Smith (2001) followed Webster's lead.

The molecular data place both *Keayodendron* and *Pseudolachnostylis* with *Bridelia* and *Cleistanthus* (Wurdack et al., in press, for *Pseudolachnostylis* only; this study). Stuppy (1996) came to the same conclusion and united these four genera in his *Bridelia* group according to their seed coat anatomy. *Bridelia*, *Cleistanthus*, and *Keayodendron* share a double disc in pistillate flowers (Radcliffe-Smith, 2001). This double disc is also described and illustrated in *Pseudolachnostylis* (Pax and Hoffmann, 1922, and P. Hoffmann's own observations). It is a potential synapomorphy of this subclade because it is not present in *Gonatogyne* and *Savia* (P. Hoffmann, unpublished data). The position of *Keayodendron* within clade F2 is at present unclear.

***Phyllanthus subgenus Kirganelia is not monophyletic***—*Phyllanthus* subgenus *Kirganelia* was proposed by Webster (1956) based on *Kirganelia* A. Juss. to accommodate species with phyllanthoid branching, five stamens, colporate pollen grains, and 3–10 carpels. He considered this variable subgenus to be primitive, comprising *P.* sections *Anisonema* and *Floribundi*. The latter section includes *P. nummulariifolius* and *P. tenellus* (Webster, 1957). Webster (1967) later described the new *P.* section *Pentandra* in subgenus *Kirganelia* to accommodate *P. nummulariifolius* and *P. tenellus* along with the type, *P. pentandrus*. He stated (Webster, 1967: 334) that ''. . . this section is significant phylogenetically because most of its taxa have precisely the habit and appearance of species of subg. *Phyllanthus*, from which they scarcely differ in anything more than the five-merous rather than three-merous androecium. Since *P. tenellus* is the only herbaceous diploid species with phyllanthoid branching, it and closely related taxa such as *P. capillipes* Schum. [= *P. nummulariifolius*] may be regarded as the nearest living equivalents of the taxa ancestral to subg. *Phyllanthus*.'' Both of Webster's studies focused on the Americas and dealt with few species of this predominantly Old World group.

Brunel (1975, 1987) studied the genus *Phyllanthus* extensively in continental Africa. He remarked on the heterogeneity of subgenus *Kirganelia* and proposed to segregate the species related to *Phyllanthus tenellus* Roxb. in a new subgenus *Tenellanthus* (Brunel, 1987) which was never validly published.

Our study included for the first time species of both *P. subgenus Kirganelia* section *Anisonema*, as well as *P. subgenus Kirganelia* section *Pentandra* (*P. subgenus Tenellanthus* section *Tenellanthus*, nomen invalidum; Brunel, 1987). The three sampled taxa of section *Anisonema* belong to a morphologically homogeneous group with a center of diversity in Madagascar. All closely resemble *P. casticum*, and characters of the constituent taxa overlap. Species identification is provisional pending a taxonomic revision (M. Ralimanana and P. Hoffmann, unpublished data).

Placement of these taxa in our analyses corroborates Brunel's (1975, 1987) view that subgenus *Kirganelia* is heterogeneous, as well as Webster's (1967) comparison of his *P.* section *Pentandra* with subgenus *Phyllanthus*. *Phyllanthus nummulariifolius* is found in a subclade with *Breynia*, *Glochidion*, and *Sauropus*, which in the *PHYC* analysis also contains *Phyllanthus epiphyllanthus* (subgenus *Xylophylla*) and *P. lokohensis* (subgenus *Phyllanthus*). Two accessions of *P. nummulariifolius* were sequenced to confirm this placement. The three accessions of subgenus *Kirganelia* section *Anisonema*

(*P.* cf. *decipiens*, *P.* cf. *fuscoluridus* and *P.* cf. *mantsakariva*) form a monophyletic group as predicted by their similar morphology. This clade is sister to *Phyllanthus calycinus* of subgenus *Isocladus* in this study with limited sampling in the largest genus of Phyllanthaceae (c. 800 species).

***Western Indian Ocean Wielandieae***—The centre of diversity for the taxa united here in clade F4 is Madagascar, with few species also represented in the Seychelles, the Comoro Islands, and the East coast of Kenya. They are morphologically similar despite being currently placed in the four different genera *Blotia*, *Petalodiscus*, *Savia* and *Wielandia*. The type of *Savia* is from Hispaniola and belongs in clade F2 as sister to *S. dictyocarpa* sampled here (Wurdack et al., in press). This shows the degree of taxonomic confusion surrounding this poorly known group. The lack of support for the internal nodes in this clade indicates that generic boundaries are in need of revision. The entire clade is revised in a forthcoming publication (Hoffmann and McPherson, in press).

***Conclusions***—Results of DNA sequence analyses using *matK* and *PHYC* correspond well with each other and with those separately obtained from analysis of *rbcL* (Wurdack et al., in press). Inclusion of missing genera and sampling of more taxa in problematic genera, namely *Cleistanthus* and *Phyllanthus*, as well as increasing the number of plastid markers analyzed and sequencing a longer fragment of low-copy nuclear *PHYC* may refine the phylogenetic hypothesis presented here.

*Note added in proof*: The high level of sequence divergence observed in the two accessions of *Drypetes madagascariensis* (outgroup taxa) may possibly be due the amplification of different *PHY* paralog, *PHYE* (in one of the accessions), instead of *PHYC*, which is otherwise used in our analyses.

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