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IDENTIFYING CLADES IN ASIAN ANNONACEAE: MONOPHYLETIC GENERA IN THE POLYPHYLETIC MILIUSEAE[1]

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The tribe Miliuseae (Annonaceae) comprises six genera distributed in Asia: *Alphonsea*, *Mezzettia*, *Miliusa*, *Orophea*, *Platymitra*, and *Phoenicanthus*. A phylogenetic study to investigate the putative monophyly of the tribe and the intergeneric relationships is presented here. Nucleotide sequences of the plastid gene *rbcL*, *trnL* intron, and *trnL-trnF* intergenic spacer were analyzed from 114 Annonaceae taxa, including 24 Miliuseae species and two outgroups using maximum parsimony and Bayesian inference. The two data sets (*rbcL* and the *trnL-trnF* regions) were analyzed separately and in combination. Miliuseae were found to be polyphyletic due to the position of *Mezzettia* and are part of a large, predominantly Asian and Central-American clade (miliusoid clade). Although intergeneric relationships were poorly resolved, all genera, except *Polyalthia*, were monophyletic, supporting previous generic delimitation based on morphology. A group of three *Polyalthia* species seems the most likely sister group of *Miliusa*. Several infrageneric groups of *Miliusa*, *Orophea*, and *Polyalthia* are supported by both molecular and morphological data. No morphological synapomorphies have yet been found for the miliusoid clade. Molecular clades within the miliusoid clade, however, can be characterized by size and the shape of the outer petals, number of ovules per carpel, and the size of the fruits.

Key words: Annonaceae; Bayesian inference; maximum parsimony; *Miliusa*; Miliuseae; *rbcL*; *trnL* intron; *trnL-trnF* intergenic spacer.

Annonaceae (soursop family) are a pantropical family of shrubs, trees, and lianas, consisting of ca. 130 genera and 2300 species. The family is a member of Magnoliales and basally positioned within the angiosperms together with Canellales, Laurales, Piperales, Chloranthales, and monocots (Soltis et al., 2000; APG II, 2003). Sister to Annonaceae are Eupomatiaceae (Qiu et al., 2000), which were previously included as a subfamily within Annonaceae and have always been considered closely related based on morphological characters (e.g., karyology; Morawetz, 1988).

Although the position of Annonaceae among the flowering plants and their monophyly are not disputed (e.g., Fries, 1959; Keßler, 1993; Doyle et al., 2000), the relationships of the genera within the family are not well understood. Morphological characters useful for the delimitation of genera and species have overlap at higher taxonomic levels (e.g., tribal level). More or less formal classifications based on intuition or phenetic analysis of morphological characters (Hutchinson, 1923, 1964; Sinclair, 1955; Fries, 1959; Walker, 1971; Van Heusden, 1992; Van Setten and Koek-Noorman, 1992; Keßler, 1993; Koek-Noorman et al., 1997) do not accurately predict relationships between genera in Annonaceae.

DNA sequence data provide an alternative source of characters suitable for building a phylogenetic classification of the family, but few molecular studies have been performed on Annonaceae. Van Zuilen (1996) and Meade (2000) used molecular data (RAPDs, RFLPs, the *trnL-trnF* intergenic spacer, and ITS sequences) to establish relationships within and between a small number of selected genera in Annonaceae. The only comprehensive molecular study on the family level was carried out by Bygrave (2000; also partly published in Doyle et al., 2000) on 130 Annonaceae taxa collected worldwide. Using only *rbcL* gene sequence data, he was able to resolve many suprageneric relationships, but other relationships among and within genera remained unresolved.

This study focuses on the Asian genus *Miliusa* A. DC., which comprises approximately 35 species in the lowland forests from Southeast Asia to Australia and New Guinea. Some species are confined to drier, monsoon-affected areas and are deciduous. The genus is generally characterized by strongly pubescent receptacles, outer petals similar to the sepals, inner petals predominantly with a saccate base, and stamens with a connective not extending over the theca (the so-called miliusoid stamen type, Fig. 1a). During the revision of the *Miliusa* species of the Flora Malesiana region and Australia (Mols and Keßler, 2003b), it became evident that not all *Miliusa* species had inner petals with a saccate base (i.e., *M. amplexicaulis* Ridl. and *M. parviflora* Ridl.). Additionally, *M. amplexicaulis* differs from the other *Miliusa* species in having a thick arc of glandular tissue at the base instead of running along the midrib. These morphological differences might warrant the exclusion of these species and related taxa from *Miliusa*.

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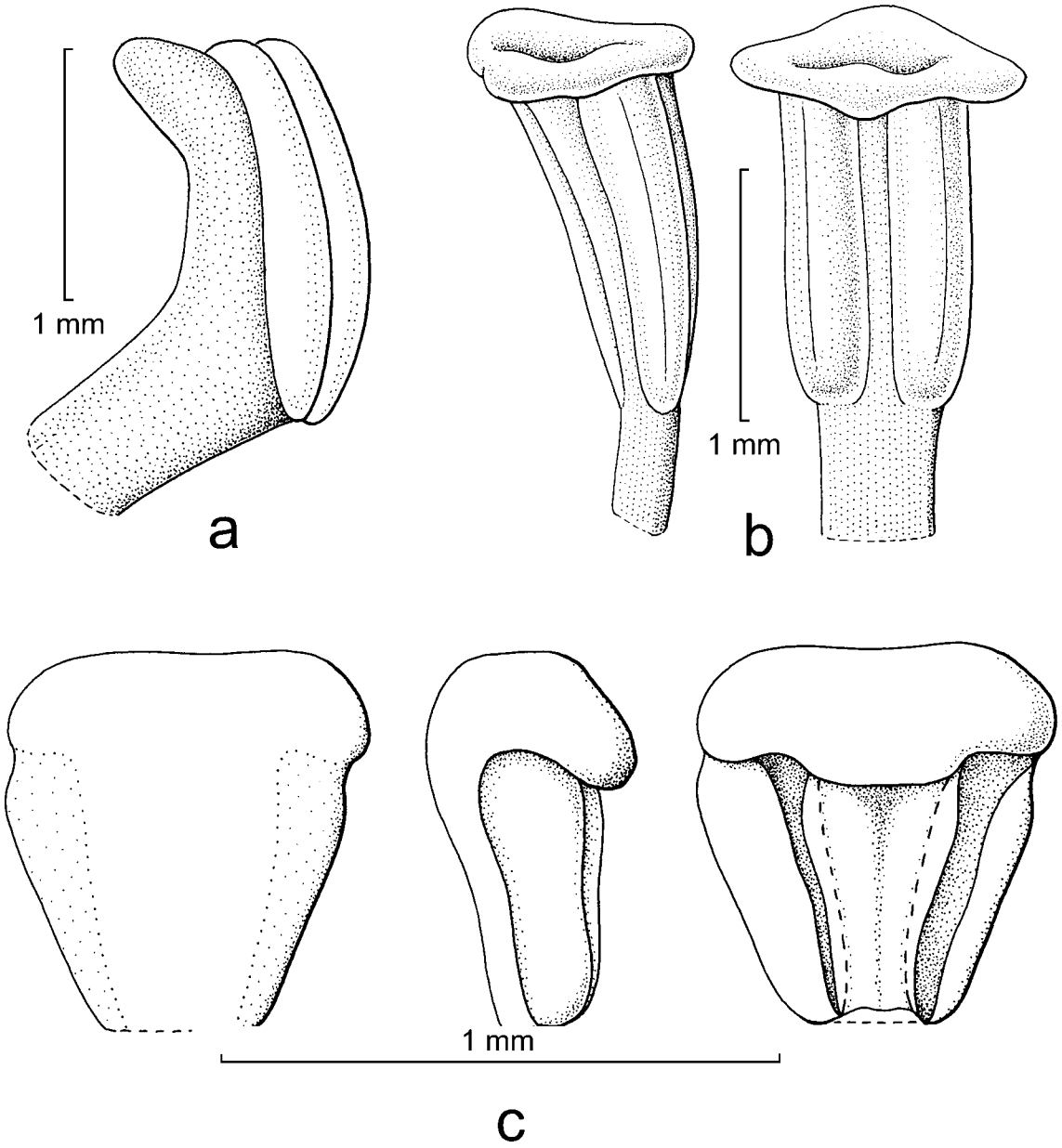

Fig. 1. Stamen types within the Annonaceae. (a) Miliusoid (lateral view; *Miliusa velutina* (Dunal) Hook. f. & Thomson). (b) Uvarioid (lateral [left] and abaxial [right] side; *Phaeanthus nutans* Hook. f. & Thomson). (c) Introrse stamen with a single pollensac (from left to right the abaxial, lateral, and adaxial sides; *Mezzettia umbellata* Becc.).

Miliusa has previously been placed in the tribe Miliuseae together with five other Asian genera: *Alphonsea*, *Mezzettia*, *Orophea*, *Phoenicanthus*, and *Platymitra* (Keßler, 1993). In total, these genera comprise approximately 130 species, which is 6% and 13% of Annonaceae species worldwide and in Asia, respectively. Their diagnostic characters are valvate sepals and petals, a low number of stamens and carpels, and the miliusoid stamen type (Fig. 1a, with the exception of *Mezzettia*; Hooker and Thomson, 1855; Keßler, 1993). The more general stamen type in Asian Annonaceae is the uvarioid type (Fig. 1b). The genera within the tribe are distinguished by the size of the outer petals (equal to the inner petals or to the sepals), fusion of the sepals (connate at the base or free), shape of the inner petals (mitre-shaped or ovate and with or without a saccate base and glandular ring), number of stamens (less than 10 or more than 20), number of ovules (1–2 or more), size of the fruits (up to 1 cm or larger) and presence of a stipe (Mols and Keßler, 2003a). In previous publications the name Saccopetaleae has been used for this tribe, but due to nomenclatorial rules and decisions the name Miliuseae should be used in stead (Mols and Keßler, 2003b). The relationships among the genera in Miliuseae have not previously been examined in a phylogenetic context.

The main objectives of this study were to investigate: (1) whether Miliuseae are monophyletic; (2) whether *Miliusa* is monophyletic and which are its sister groups; and (3) which morphological characters are phylogenetically informative in Miliuseae.

To answer these questions, the gene *rbcL*, the intron of *trnL*, and the intergenic spacer between *trnL* and *trnF*, all belonging to the plastid genome, were chosen as phylogenetic markers. The *rbcL* gene encodes for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase and has been widely used for phylogenetic reconstruction at the family level or above in angiosperms (e.g., Chase et al., 1993; Ingrouille et al., 2002). The *trnL* (UAA) intron (a group I intron) and the *trnL-trnF* intergenic spacers are noncoding regions (Gielly and Taberlet, 1994, 1996). These markers have been used in recent studies in Annonaceae (Bygrave, 2000; L. W. Chatrou et al., unpublished data), enabling us to take advantage of the large database of sequences already available.

MATERIAL AND METHODS

Taxon sampling—Taxon sampling was based on the study of Bygrave (2000) in which major clades were found within Annonaceae. All members of Miliuseae were found in his unsupported group 2, referred to as the malmeoid, piptostigmoid, miliusoid (MPM) clade after Doyle and Le Thomas (1994, 1996). This MPM clade in the study by Bygrave contained Asian, African, and American taxa. To determine the position of *Miliusa* in Miliuseae and to determine whether the tribe is monophyletic, 114 taxa were analyzed (see Appendix Supplemental Data accompanying online version of this article). These taxa include seven species of *Miliusa* (sampling based on availability of material suitable for DNA extractions, but covering the morphological and geographical variation within the genus) and four of the five other genera of Miliuseae (no suitable material of *Phoenicanthus* was available). In addition, more than 60 representatives of other Asian, African, and American taxa were included as molecular (Bygrave, 2000; L. W. Chatrou et al., unpublished data) and morphological data suggested a close relationship of these genera with Miliuseae. Finally, three *Melodorum* accessions were added as representatives of another, more distantly related, large clade found in the family cladogram (Bygrave, 2000; L. W. Chatrou et al., unpublished data).

Outgroup choice—Outgroups were species of *Anaxagorea* based on the placement of this genus as closest sister to all other Annonaceae using molecular (L. W. Chatrou et al., unpublished data; Doyle et al., 2000), biogeographical (Maas and Westra, 1984, 1985), and morphological (Doyle and Le Thomas, 1994, 1996) characters.

DNA extraction, PCR amplification, and sequencing—Total genomic DNA was extracted using either a modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) or the DNeasy Plant Mini Kit (QIAGEN, Leusden, Netherlands). The CTAB extraction was done by adding 1300 μL of CTAB (65°C) and 13 μL 2-mercaptoethanol to 50 mg of dried leaf material and homogenizing this with a micropestle in a preheated mortar. A total of 1 mL of this solution was heated to 65°C in a waterbath. After 20 min, 1 mL of chloroform : isoamylalcohol (24 : 1) was added. The sample was left to shake for 90 min and then centrifuged at 13 000 rpm for 10 min. The supernatant (300 μL) was cleaned and precipitated using the Wizard PCR Preps DNA Purification System or Wizard DNA Clean-Up System (Promega, Leiden, Netherlands). All DNA was extracted from silica-gel-dried leaves collected in the field or in botanical gardens.

The *rbcL* gene was amplified using the following two primer combinations: 1F/724R and 636F/1460R (Fay et al., 1997). The *trnL* intron and *trnL-trnF* intergenic spacer were amplified using the primer combinations c/d and e/f, respectively (Taberlet et al., 1991). The thermal cycling protocol comprised 28 cycles, each with 1 min denaturation at 94°C, 1 min annealing at 50°C, an extension of 2 min at 72°C, concluding with an additional extension of 10 min at 72°C. All PCR products were cleaned using a QIAquick PCR Purification Kit (QIAGEN). The samples were sequenced with the PCR primers and electrophoresed on an ABI 377 automatic sequencer (Applied Biosystems, Nieuwekerk a/d IJssel, Netherlands).

Phylogenetic analysis—Sequences of *trnL-trnF* were aligned automatically with the Clustal method in Megalign 4.03 (DNAstar, 1999, Madison, Wisconsin, USA) and subsequently adjusted by hand (according to the recommendation of Kelchner, 2000), resulting in the removal of 43 characters (mainly mononucleotide repeats) due to ambiguous alignment. All DNA sequences were deposited in Genbank (see Appendix in Supplemental Data accompanying the online version of this article). Aligned data matrices have been submitted to TreeBASE (study accession number: S1034; matrix accession number: M1757) and can be obtained upon request from the corresponding author.

Phylogenetic analyses were performed with PAUP* version 4.0b10 (Swofford, 2002) using maximum parsimony (MP) with the heuristic search option (10 times simple addition). The options tree bisection-reconnection (TBR), accelerated transformation optimization (ACCTRAN), and save all minimal trees (MULTREES) were invoked. Because a large number of trees were found and the memory limit quickly reached, an additional heuristic search using 1000 replicates with 10 trees saved per replicate was performed to investigate whether shorter trees could be found; when the latter was the case, one of the shorter trees was used as a starting tree for a new heuristic search. Character states were specified as unordered and equally weighted (Fitch, 1971). Indels were coded as single characters (absence or presence). Insertions/deletions were treated as missing data.

The MP analyses were performed with three different data sets: the *rbcL* data, the *trnL-trnF* data, and both data sets combined. The separate *trnL-trnF* units have been combined in one data set in stead of separate ones because the different units form a connected noncoding region. This makes it possible to compare the noncoding vs. coding region. Bootstrap analyses were performed to evaluate internal support (Felsenstein, 1985). Bootstrap percentages (BP) were calculated using 2000 replicates, saving one tree per replicate (Hedges, 1992). Because uninformative characters can lower the BP (Harshman, 1994), only the informative characters were included. Bremer support (Bremer, 1998; Donoghue et al., 1992) was calculated using AutoDecay 4.0.2 (Eriksson, 1998) with 100 addition sequence replicates per run, for the combined analysis only. The separate data sets were tested for congruence with the partition homogeneity test (PHT; Farris et al., 1995; 1000 replicates, 10 trees saved per replicate). However, as this type of test is said to be unreliable (Yoder et al., 2001; Reeves et al., 2001), the separate bootstrap trees were compared for ''hard'' incongruence (i.e., BP $\geq$ 85%; Seelanan et al., 1997; Wiens, 1998) as well. Transition/transversion (ts/tv) ratios were calculated on one of the most parsimonious trees (arbitrarily chosen).

The combined data set without the binary indels was also analyzed using Bayesian inference (BA) with MrBayes version 3.0b (Huelsenbeck and Ronquist, 2001 and references). This analysis was performed to test the robustness of the evolutionary signal in the data set, because it is based on different optimality criteria and parameter settings used in phylogeny reconstruction in comparison with MP. For the BA, the data set was split into four partitions (*rbcL*, noncoding part downstream of *rbcL*, *trnL* intron, *trnL-trnF* intergenic spacer). A DNA substitution model was assigned to each partition using MrModeltest version 1.1b (a simplified version of Posada and Crandall's [1998] Modeltest version 3.06; Nylander, 2002). For those partitions for which a model was set using a gamma (Γ)-distributed rate variation across sites, the α-value was set to unlinked. Furthermore, a rate multiplier was enforced to constrain the average rate across the partitions to one. The Markov Chain Monte Carlo analyses (MCMC; Geyer, 1991) were run for 3 000 000 generations with four simultaneous MCMC chains to calculate the posterior probabilities (PP). Prior probabilities for all trees were equal. A random tree was used as the starting point for the analysis, and one tree per 10 generations was saved. The burn-in values were determined empirically from the likelihood values. Finally, 50% majority consensus trees were calculated together with the branch lengths and approximations of the PP for the observed bipartitions. Because MrBayes only accepts one outgroup taxon, we chose *Anaxagorea luzonensis* for this purpose. The analysis was repeated four times to assure sufficient mixing by confirming conversion to the same PP and topology.

TABLE 1. Number of steps, consistency index (CI), retention index (RI), and transition/transversion ratio (ts/tv) for each codon position in *rbcL* based upon one of >10 000 most parsimonious trees (MPTs) from the combined analysis of selected Annonaceae.

Codon position	Number of steps	CI	RI	ts/tv
1	127 (22%)	0.402	0.725	1.5
2	143 (24%)	0.350	0.738	0.4
3	318 (54%)	0.616	0.723	2.4

RESULTS

rbcL analysis—The boundaries of the *rbcL* gene and adjacent downstream noncoding region were taken from Bygrave (2000) and Olmstead et al. (1992). The final alignment had a total length of 1433 bp, of which 1394 bp belonged to the *rbcL* gene and 39 bp to the noncoding region. This noncoding region was quite variable and contained seven indels, ranging in size from 4 to 11 bp, four of which were phylogenetically informative. The *Phaeanthus splendens* and *Mitrephora celebica* accessions were omitted from this analysis, because the *rbcL* region could not be amplified for these species. Of the 1440 characters used (bp + indels), 262 were variable, 160 of which (11%) were potentially phylogenetically informative. Pairwise sequence divergence varied between 0% (in nine comparisons between taxa, e.g., *Miliusa horsfieldii* and *M. mollis*) and 5.1% (*Melodorum* cf. *fruticosum* II vs. *Anaxagorea javanica*). The ts/tv ratio was 1.5. The third codon position contributed more steps than the first and second positions and also displayed a higher consistency index (CI) (Table 1).

Phylogenetic analysis yielded >10 000 trees of length 582 (CI = 0.52, RI = 0.74; Table 2). The following clades were monophyletic (Fig. 2): Two *Miliusa* clades (A1 and A2; BP < 50%, 98%), two *Orophea* clades (B1 and B2; BP 51%, 94%), *Alphonsea* (C; BP < 50%), several *Polyalthia* clades (F1, F2, F3, F4 and S; BP 56%, 57%, 64%, <50%, 82%), *Mitrephora* (O; BP 70%), *Popowia* (P; BP 96%), *Pseuduvaria* including *Petalolophus* and *Craibella* (N; BP 84%), *Marsypopetalum* including *Polyalthia* pro parte (L; BP 72%), *Neouvaria* (G; BP 84%), Central American clade including *Stelechocarpus burahol* (K; BP < 50%), *Melodorum* (T; BP 100%), and some South American genera (SAm; BP < 50%).

trnL-trnF analysis—Boundaries of the *trnL-trnF* region were taken from *Nicotiana tabacum* L. (Solanaceae) (GenBank accession Z00044) and L. W. Chatrou et al. (unpublished data). The final alignment has a total length of 995 bp, of which 554 belong to the *trnL* intron and 441 to the *trnL-trnF* intergenic spacer. In total, 85 indels were present, of which 32 belong to the intron and 53 to the spacer. One of the largest gaps was a deletion of 14 bp found in all *Miliusa* species only, whereas another large insertion (8 bp) was shared by many taxa. Of the 1080 characters, 391 were variable, 198 of which (18%) were potentially phylogenetically informative. Pairwise sequence divergence was between 0% (in 40 comparisons between taxa, e.g., *Miliusa horsfieldii* and *M. mollis*) and 7.5% (*Melodorum* cf. *fruticosum* II vs. *Anaxagorea javanica*). The ts/tv ratio was 0.8.

Phylogenetic analysis yielded >10 000 trees of length 577 (CI = 0.78, RI = 0.86; Table 2), with none of the individual trees fully resolved. In comparison with *rbcL*, the strict consensus obtained from this analysis showed more resolution and resulted in more clades supported by high BP. The following clades were monophyletic (Fig. 3): *Miliusa* (A; BP 96%), *Orophea* (B; BP 66%), *Alphonsea* (C; BP 85%), several *Polyalthia* clades (F1, F2, F4 and S; BP 93%, 99%, 62%, and 84%), *Popowia* (P; BP 96%), *Phaeanthus* (Q; BP 77%), *Pseuduvaria* including *Petalolophus* and *Craibella* (N; BP 99%), *Marsypopetalum* including *Polyalthia p.p.* (L; BP 94%), *Neo-uvaria* (G; BP 100%), *Sapranthus* with *Tridimeris* (K; BP 72%), *Meiogyne* including *Fitzalania* (H; BP 50%), *Mitrephora* (O; BP <50%), and *Melodorum* (T; BP 100%). Compared with the *rbcL* results, five additional clades were supported: U (most Asian taxa; BP 82%), a clade combining U + *Monocarpia* (R; BP 95%), a clade V (combining U + R + SAm + S + Afr + *Cremastosperma microcarpum* + *Malmea dielsiana* + *Onychopetalum periquino* + *Pseudoxandra lucida*; BP 98%), a clade V + T (BP 83%), and a clade V + T + E (BP 100%). Compared with the *rbcL* results, clades A, B, and S included additional species.

Combined parsimony analysis—The partitions (*rbcL* including downstream noncoding region, *trnL* intron, *trnL-trnF* intergenic spacer) were not significantly incongruent (PHT, *P* = 0.67). We also investigated whether ''hard'' incongruence was present. When comparing the cladograms obtained from

TABLE 2. Values and statistics before and from both the maximum parsimony (MP) and Bayesian (BA) analyses, of the *rbcL*, *trnL-trnF*, and combined data for selected Annonaceae.

Characteristic	*rbcL*	*trnL-trnF*	Combined data MP	Combined data BA
Number of species	112	114	114	114
Number of included characters	1440	1080	2520	2428
Number of indels	7	85	92	0
Number of excluded characters	0	43	43	135
Number of variable sites	262	391	653	—
Number of phylogenetically informative sites	160	198	338	—
ts/tv ratio	279/191 = 1.5	144/176 = 0.8	—	—
Sequence divergence	0–5.1%	0–7.5%	—	—
Number of MPTs	>10 000	>10 000	>10 000	21 762
Tree length (steps)	582	577	1184	—
Consistency index (CI)	0.521	0.776	0.634	—
Retention index (RI)	0.742	0.855	0.780	—
Rescaled CI (RC)	0.386	0.664	0.495	—
Number of clades with support (BP ≥ 70%, PP ≥ 95%)	18	31	44	48
Number of clades with lesser support (BP 50–69%)	15	15	10	—

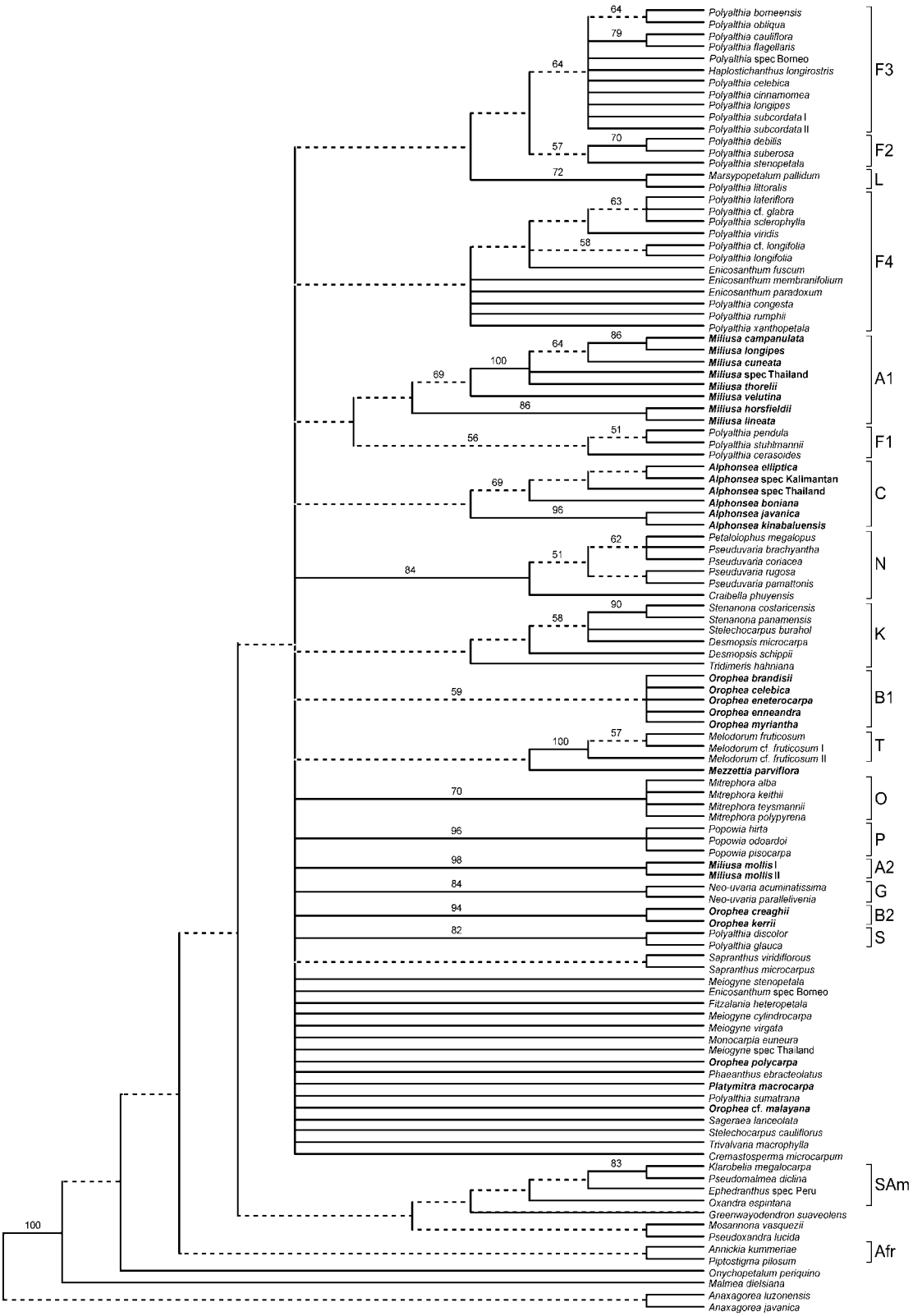

Fig. 2. Strict consensus cladogram of >10 000 most parsimonious trees (MPTs) of the *rbcL* data set for selected Annonaceae. BP > 50% are indicated above the branches. Dotted lines indicate branches with BP < 70%. Length of the MPTs = 582, CI = 0.52, RI = 0.74. Members of the tribe Miliuseae are indicated in boldface type. *Phaeanthus splendens* and *Mitrephora celebica* are missing due to amplification problems. SAm = South American origin, Afr = African origin.

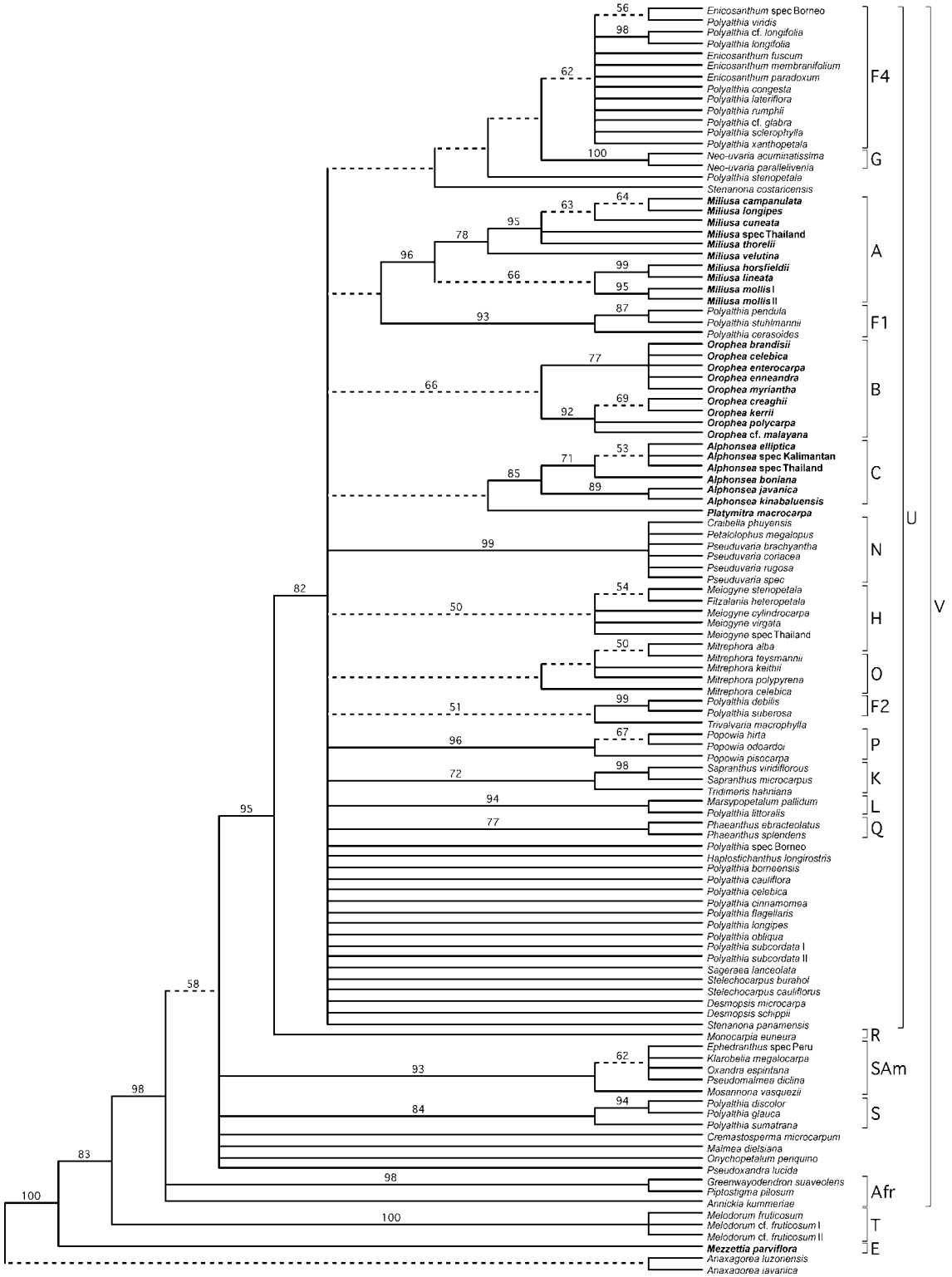

Fig. 3. Strict consensus cladogram of >10 000 MPTs of the *trnL-trnF* data set for selected Annonaceae. BP > 50% are indicated above the branches. Dotted lines indicate branches with BP < 70%. Length of the MPTs = 577, CI = 0.78, RI = 0.86. Members of the tribe Miliuseae are indicated in boldface type. SAm = South American origin, Afr = African origin.

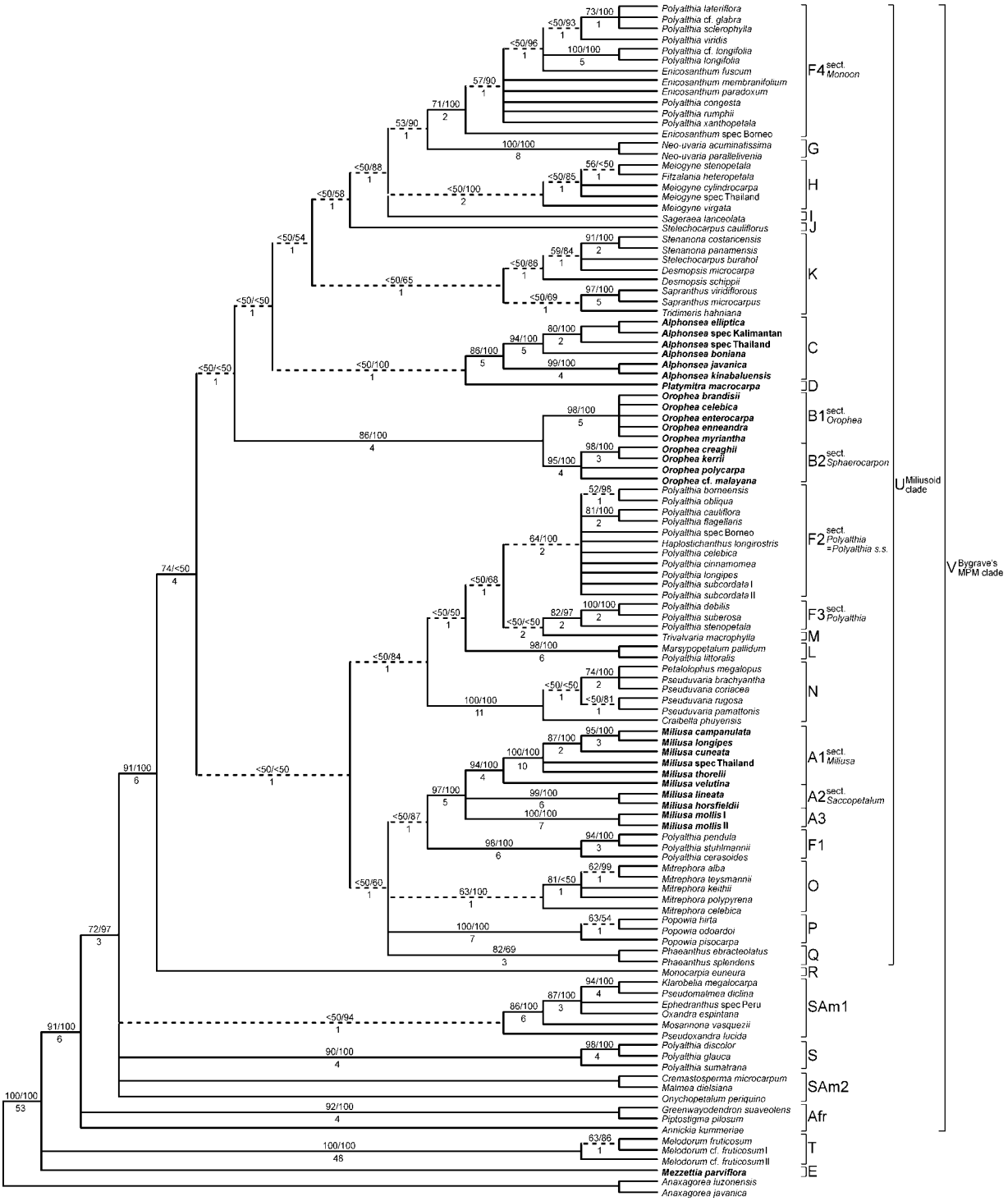

Fig. 4. Strict consensus cladogram of >10 000 MPTs of the combined data set. BP (left of slash) and PP (right of slash) are indicated above the branches. Numbers below the branches are decay indices. Dotted lines indicate branches with BP < 70%. Length of the MPTs = 1184, CI = 0.63, RI = 0.78. Members of the tribe Miliuseae are indicated in boldface type. SAm = South American origin, Afr = African origin.

the analysis of each region several differences were found, but none of these were supported by high (≥85%) BP.

The combined data set comprised a total of 2520 characters, including 92 indels. Of these characters, 653 were variable, 338 of which (13%) were potentially phylogenetically informative. Phylogenetic analysis yielded >10 000 MPTs of length 1184 (CI = 0.63 and RI = 0.78; Table 2).

Bootstrap analysis of this combined data set provided more supported clades than either individual data set (Fig. 4). The following genera were recovered: *Miliusa* (A1 + A2 + A3;

BP 97%), *Orophea* (B1 + B2; BP 86%), *Alphonsea* (C; BP 86%), several *Polyalthia* clades (F1–F4 [including *Enicosanthum*] and S; BP 98%, 64%, 82%, 71%, and 90%), *Mitrephora* (O; BP 63%), *Popowia* (P; BP 100%), *Phaeanthus* (Q; BP 82%), *Pseuduvaria* including *Petalolophus* and *Craibella* (N; BP 100%), *Marsypopetalum* including *Polyalthia p.p.* (L; BP 98%); *Neo-uvaria* (G; BP 100%), *Meiogyne* including *Fitzalania* (H; BP $<$ 50%); Central American clade including *Stelechocarpus burahol* (K; BP $<$ 50%); and *Melodorum* (T; BP 100%). Compared with the *rbcL* and *trnL-trnF* results, several clades (A1–A3, B1, B2, C, N) were well resolved at the species level.

Combined Bayesian analysis—For the *rbcL* data set, the hierarchical likelihood test indicated the general time reversible model (Tavare, 1986) with a gamma-distributed rate and an invariable proportion of sites (GTR + Γ + I) to be the least-rejected model. The noncoding region downstream of the *rbcL* gene was assumed to evolve under the Jukes-Cantor model (Jukes and Cantor, 1969) with a gamma-distributed rate only (JC + Γ). In both *trnL-trnF* partitions, the DNA sequence evolution was best explained using the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with a gamma-distributed rate (HKY + Γ).

Of the 30 000 trees obtained from BA the burn-in was estimated, resulting in more than 7500 trees being discarded. The remaining trees (Table 3) yielded a consensus tree (not shown here, PP given on strict consensus of the MP analysis: Fig. 4) with a topology similar to the bootstrap consensus tree of the combined analysis. Three clades with BP $\geq$ 70% did not receive high PP: Q (PP 69%), U (PP $<$ 50%), and an internal clade of O (PP $<$ 50%). Seven additional clades obtained strong support (PP $\geq$ 95%) in BA only: placement of *Platymitra* (D) as sister to *Alphonsea* (C; PP 100%), *Meiogyne* including *Fitzalania* (H; PP 100%), *Mitrephora* (O [and an internal clade]; PP 100%, 99%), and several (internal) *Polyalthia* clades (F2 and F4; PP 100%, 98%, 96%).

DISCUSSION

Phylogenetic utility of rbcL vs. trnL-trnF—The two DNA regions sequenced show differences in resolution. The combined *trnL* intron and *trnL-trnF* intergenic spacer proved to be useful for discovering some intergeneric relationships because these regions appeared less conserved (18% of the characters were informative) and showed little homoplasy (CI = 0.855). Using the *rbcL* gene alone, we were not able to clarify any intergeneric relationships due to some conflict in the data (CI = 0.521) and lack of informative characters (only 11% of the characters were informative). As is generally found (Källersjö et al., 1998), most of the changes within *rbcL* were in the third codon position. These changes were also phylogenetically informative as the less conservative third codon positions did not show more homoplasy than the first and second positions (Table 1). Therefore, the third codon positions were not excluded from the analyses as this resulted in a loss, and not gain, of phylogenetic information (data not shown). The combined *trnL-trnF* and *rbcL* data set led to more resolution and higher bootstrap values (Table 2). Because only a limited number of potentially phylogenetically informative characters were present to infer the relationships among the 114 taxa, analyses with a smaller number of taxa (mainly focusing on the Miliuseae) were performed. These analyses did not result in a better resolved cladogram and/or higher bootstrap percentages (data not shown).

Polyphyly of Miliuseae—The five genera formerly assigned to Miliuseae do not form a clade, as *Mezzettia* (E) is found outside clade U near the outgroup. This genus was only tentatively placed in Miliuseae (Keßler, 1993) based on the reduced number of carpels and stamens, which also occur in *Orophea* and *Phoenicanthus* (Van der Heijden and Keßler, 1990). The genus lacks the typical miliusoid stamen type considered to be the most important diagnostic character of the tribe. Instead, the stamens in this genus are peculiar in having introrse thecae with a single sporangium (Fig. 1c). Other characters also suggest an isolated position of *Mezzettia* in Annonaceae. Its seeds have three integuments, which are only found in a few other taxa in the family (Christmann, 1986, 1989). Furthermore, the satellite chromosomes have a small satellite (Okada and Ueda, 1984), whereas in all other Annonaceae they are large. Additionally, Okada and Ueda (1984) observed $2n = 14$ for *Mezzettia parviflora*, which is only found in nine annonaceous genera worldwide (Morawetz, 1986; Morawetz and Le Thomas, 1988). Three of these nine taxa belong to a group referred to as the ambavioids (Doyle and Le Thomas, 1994, 1996), which are characterized by the presence of three integuments in the seeds, chromosome number $x = 7$, monosulcate pollen, and irregular endosperm ruminations. The last two characters are also shared by *Anaxagorea*, which is considered the sister group of the ambavioids. Molecular and morphological characters indicate that *Mezzettia* is part of the ambavioid group, which is placed as sister to the rest of Annonaceae (except *Anaxagorea*).

The genera *Alphonsea*, *Orophea*, and *Platymitra* can neither be confirmed nor rejected as members of Miliuseae. Few of the generic relationships are supported by high bootstrap percentages. Until more resolution is found, clade U (Fig. 4) is considered to be the clade containing the genus *Miliusa* and its closest relatives. Because we do not want as yet to give this large clade formal taxonomic status, it will be referred to as the miliusoid clade. The non-Miliuseae genera in this clade were previously ascribed to several other tribes or groups (Keßler, 1993). The miliusoid clade consists of 24 genera (Fig. 4), but further research is needed to elucidate the relationships among these genera.

Monophyly of Miliusa—For *Miliusa* (A), seven of the approximately 35 species have been included. These species end up in three clades (A1–A3, Fig. 4). Clade A1 consists of five species, which differ only slightly in number of ovules, shape and size of the leaves, and apices of the inner petals. *Miliusa velutina* differs from the other included species in its deciduous habit, shape and size of the leaves, sepals and petals, fruits, and number of stamens and carpels. It much more resembles the species from clade A2, *M. horsfieldii* and *M. lineata*, especially in the size and shape of the fruits. However, the placement of *M. velutina* as the sister to the rest of A1 and not in A2 corresponds with original sections recognized in *Miliusa*. All species in clade A1 have one or two ovules (section *Miliusa*), whereas the species in A2 have more than two ovules (section *Saccopetalum*). Additionally, *M. velutina* is one of the few species in the genus [together with *M. parviflora* and *M. andamanica* (King) Finet & Gagnep.] with non-saccate inner petals. This indicates that the species with non-saccate inner petals should be included in the genus. *Miliusa horsfieldii* and

M. lineata (A2) had identical sequences (only differing in the number of base pairs we were able to obtain). This supports the view that the morphologically almost identical *M. lineata* and several other species should be synonymized with *M. horsfieldii* (Mols and Keßler, 2003b).

The third clade A3 contains two specimens of *Miliusa mollis* var. *mollis* and forms an unresolved branch with the other *Miliusa* clades. This species is a member of a group of six taxa (*M. amplexicaulis*, *M. fusca* Pierre, *M. glandulifera* C. E. C. Fischer, *M. glochidioides* Hand.-Mazz., *M. mollis* var. *mollis*, and *M. mollis* Pierre var. *sparsior* Craib), which deviate from the other *Miliusa* species in shape of the inner petals (broadly ovate to almost triangular, with a basal glandular tissue ring, and without a saccate base) and connate sepals (J. B. Mols, personal observation). This study shows that these species should be included in *Miliusa*.

The most likely sister group of *Miliusa* based on both MP and BI analysis is clade F1 containing three *Polyalthia* species. However, bootstrap support is low and no apparent morphological similarities between clade F1 and *Miliusa* are known.

Monophyly of other genera—Although most intergeneric relationships were not clarified, we found that most genera are monophyletic and supported by high bootstrap percentages as far as can be concluded on the basis of a limited sampling for some of the genera. Only three genera appear to be not monophyletic. For two genera, *Stelechocarpus* and *Desmopsis* (found in clades J and K), the polyphyly does not receive strong support.

The only genus with high support for polyphyly is *Polyalthia*, which is one of the largest genera among Asian Annonaceae, consisting of approximately 150 species. Generic delimitation has been based on sepal and petal aestivation, shape, and size. Several authors (e.g., Rogstad and Le Thomas, 1989; Schatz and Le Thomas, 1990; Van Setten and Koek-Noorman, 1992; Doyle and Le Thomas, 1994; Johnson and Murray, 1999) have suggested that *Polyalthia* should be split into several smaller genera. This has not been done because no suitable characters were found to easily distinguish ''natural groups.'' In total, 28 species of *Polyalthia* were included in this study, among which 26 were from Asia, one from Madagascar (*P. pendula*), and one from Africa (*P. stuhlmannii*). Additionally, one species of the African genus *Greenwayodendron* was included, which previously has been considered a separate section within *Polyalthia* (e.g., Fries, 1959). In total, seven clades (F1–F4, L, S, and Afr) were found, containing species assigned to *Polyalthia* (Fig. 4).

Greenwayodendron is situated as sister to the African genus *Piptostigma* (Afr) and seems not closely related to the other clades containing species of *Polyalthia*, warranting its status as a separate genus. The second *Polyalthia* clade (S) contains three species that are part of what Rogstad (1989) referred to as the *Polyalthia hypoleuca* complex, which share several unique morphological characters (concerning the bark, leaves, and seeds) not found elsewhere within *Polyalthia*. Our results indicate that this complex of species should be transferred to a new genus. The placement of this complex outside clade T among the South-American genera (SAm1 + 2) is supported by pollen morphology, because all members of clades S, Sam 1, and Sam 2 have monosulcate, boat-shaped pollen (Rogstad and Le Thomas, 1989).

The remaining five clades of *Polyalthia* species are all part of clade U. Clade F1 is the weakly supported sister group of *Miliusa* in the combined analyses. It consists of three species. The African species *P. stuhlmannii* and *P. pendula* are similar in their sepals, stamens, number of ovules, fruits, and seeds. A difference is found in the pollen as *P. pendula* is described as inaperturate (Schatz and Le Thomas, 1990) and *P. stuhlmannii* as monosulcate (Le Thomas, 1988; Doyle and Le Thomas, 1996). The Asian *P. cerasoides* differs mainly from these two species in having smaller flowers that are always solitary, smaller globose fruits, and disulculate pollen (Le Thomas, 1988).

Clade F2, containing the type species *P. subcordata*, should be considered as *Polyalthia* sensu stricto (s.s.). The genus *Haplostichanthus*, which is nested within this clade, consists of only six species found in the Philippines, Moluccas, New Guinea, and Australia. In her revision of the genus, Van Heusden (1994b) indicated a close relationship with some *Polyalthia* species based on leaf and flower characters.

Clade F3 is closely related (though with low branch support) to clade F2. It contains only three species, but two of these, *Polyalthia debilis* and *P. suberosa*, are quite similar in their habit, petals, and fruits. The close relationship between clades F2 and F3 is supported by morphology because all the species have two or more ovules, which is a key character for *Polyalthia* section *Polyalthia*.

In clade F4, besides *Polyalthia* species, several species of *Enicosanthum* are present. The latter includes a large number of species formerly assigned to *Polyalthia*. *Enicosanthum* and *Polyalthia* are similar in general appearance and fruit and seed characters. The main reason of segregating the genus is the presence of imbricate sepals and petals in *Enicosanthum* and valvate sepals and petals in *Polyalthia*. The results presented here indicate that *Polyalthia* species of clade F4 should be included in *Enicosanthum* and that when dealing with the classification of *Polyalthia* more emphasis should be placed on fruit rather than floral characters. Clade F4 can be distinguished from clades F2 and F3 by the presence of a single ovule (*Polyalthia* section *Monoon*).

Clade L consists of *P. littoralis* and *Marsypopetalum*. According to Van Heusden (1992) *P. crassa* R. N. Parker (and related species, i.e., *P. littoralis* and *P. modesta* (Pierre) Finet & Gagnep.; P. J. A. Keßler, personal observation) might be erroneously placed in *Polyalthia* because it clearly resembles *Marsypopetalum* due to similarity of the leaves, perianth, stamens, and fruits. According to all analyses, *Polyalthia littoralis* (and relatives) should be transferred to *Marsypopetalum*.

According to our results, *Pseuduvaria* (N) also contains the monotypic genus *Petalolophus* endemic to New Guinea. *Petalolophus megalopus* is unique in having large winged inner petals. *Pseuduvaria* flowers are generally unisexual. In contrast, *Petalolophus* has bisexual flowers and, larger inner petals. However, *Pseuduvaria novaguineensis* J. Sinclair has long peduncles, short pedicels with an articulation in the middle, bisexual flowers, dissimilar outer and inner stamens, and smooth, globose monocarps just as *Petalolophus megalolopus* (Su, 2002), which supports the molecular placement of *Petalolophus* in *Pseuduvaria*.

Monotypic *Fitzalania*, endemic to Australia, is placed within *Meiogyne* (H). Based on size and shape of inner and outer petals and position of the lateral ovules, the genus was placed in an informal group together with *Orophea*, *Platymitra*, *Popowia*, *Pseuduvaria*, and *Petalolophus* (Van Heusden, 1992). In contrast, endosperm ruminations and number of seeds places *Fitzalania* together with, among others, *Haplostichanthus*,

Desmopsis, *Polyaulax*, *Oncodostigma*, *Guamia*, and *Ancana* (Van Setten and Koek-Noorman, 1992). The last four genera are now included in *Meiogyne* (Van Heusden, 1994a). Van Heusden indicated that it was not warranted to include *Fitzalania* in *Meiogyne* based on the floral differences. Our results, however, show *Fitzalania* to be nested within *Meiogyne*, although support for this is low, suggesting that seed characters may be more informative in this case.

The miliusoid clade also contains a clade K, consisting of endemic Central American genera. That this clade is placed among Asian taxa suggests multiple invasions of Central America, at least once by species from more widespread Neotropical genera such as *Annona* and *Guatteria* and by endemic Central American taxa derived from Asian ancestry. This Asian and Central American link for Annonaceae was also suggested by Schatz (1987). He refers to Gentry (1982a, b) and several others who describe remnants of a small tropical Laurasian floristic element in Central America.

Monophyly of subgenera—Besides the monophyly of the genera of the Miliusoid clade, several well-supported infrageneric relationships were found. *Orophea* consists of approximately 50 species, and Keßler (1988) recognized two subgenera. Subgenus *Orophea* contains approximately 30 species characterized by the presence of an indument on the young shoots and carpels, six ovules per carpel, and cylindrical fruits and seeds. Subgenus *Sphaerocarpon* Keßler (including the genus *Mezzettiopsis* Ridl.; Leonardia and Keßler, 2001) consists of 22 species characterized by the absence of indument on the young shoots and carpels, two ovules per carpel, and globose fruits and seeds. In this study, two subclades can be recognized, clade B1 consisting of *O. brandisii*, *O. celebica*, *O. enterocarpa*, *O. enneandra*, and *O. myriantha*, and clade B2 containing *O. creaghii*, *O. kerrii*, *O. polycarpa*, and *O.* cf. *malayana* (Fig. 4). Clade B1 corresponds with subgenus *Orophea* and clade B2 with subgenus *Sphaerocarpon*. Leonardia and Keßler (2001) performed a phylogenetic analysis of the latter subgenus based on morphological characters and showed that subgenus *Sphaerocarpon* is monophyletic, which is supported by our molecular analysis.

The original members of Miliuseae were almost all found in a clade consisting of mainly Asian, Central-American, and some African taxa (miliusoid clade: U), which were previously ascribed to other tribes within Annonaceae. Miliuseae cannot be considered monophyletic as *Mezzettia* previously belonging to this tribe, is positioned outside the miliusoid clade, and several new taxa need to be included. The intergeneric relationships in the miliusoid clade remained poorly resolved, but the genera included, except *Polyalthia*, appear to be monophyletic. The genus *Miliusa* was found to be monophyletic and includes the nonsaccate species. A tentative sister group of *Miliusa* seems to be a group of three *Polyalthia* species, which most likely represents a much larger number of *Polyalthia* species, from East Africa, Madagascar (Schatz and Le Thomas, 1990), and mainland Asia. In *Miliusa*, *Orophea*, and *Polyalthia*, the molecular clades found are congruent with sectional divisions. It can be concluded that molecular data support the previous generic and intrageneric delimitation based on morphology. Pollen, fruit, and seed characters, and number of ovules per carpel especially appear to be phylogenetically informative and promising for the elucidation of the intergeneric relationships of the miliusoid clade.

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