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PHYLOGENETIC ANALYSES OF CORNALES BASED ON 26S RRNA AND COMBINED 26S RDNA-*MATK*-*RBCL* SEQUENCE DATA[1]

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Nuclear 26S rDNA sequences were used to corroborate and test previously published *matK-rbcL*-based hypotheses of phylogenetic relationships in Cornales. Sequences were generated for 53 taxa including *Alangium*, *Camptotheca*, *Cornus*, *Curtisia*, *Davidia*, *Diplopanax*, *Mastixia*, *Nyssa*, and four families: Grubbiaceae, Hydrangeaceae, Hydrostachyaceae, and Loasaceae. Fifteen taxa from asterids were used as outgroups. The 26S rDNA sequences were initially analyzed separately and then combined with *matK-rbcL* sequences, using both parsimony and maximum likelihood methods. Eight strongly supported major clades were identified within Cornales by all analyses: *Cornus*, *Alangium*, nyssoids (*Nyssa*, *Davidia*, and *Camptotheca*), mastixioids (*Mastixia* and *Diplopanax*), Hydrangeaceae, Loasaceae, *Grubbia*-*Curtisia*, and *Hydrostachys*. However, relationships among the major lineages are not strongly supported in either 26S rDNA or combined 26S rDNA-*matK-rbcL* topologies, except for the sister relationships between *Cornus* and *Alangium* and between nyssoids and mastixioids in the tree from combined data. Discrepancies in relationships among major lineages, especially the placement of the long-branched *Hydrostachys*, were found between parsimony and maximum likelihood trees in all analyses. Incongruence between the 26S rDNA and *matK-rbcL* data sets was suggested, where Hydrangeaceae was found to be largely responsible for the incongruence. The long branch of *Hydrostachys* revealed in previous analyses was reduced significantly with more sampling. Maximum likelihood analysis of combined 26S rDNA-*matK-rbcL* sequences suggested that *Hydrostachys* might be sister to the remainder of Cornales, that *Cornus*-*Alangium* are sisters, that nyssoids-mastixioids are sisters, and that Hydrangeaceae-Loasaceae are sisters, consistent with previous analyses of *matK-rbcL* sequence data.

Key words: Cornales; Grubbiaceae; Hydrostachyaceae; incongruence; long-branch attraction; *matK-rbcL*; phylogenetics; 26S rDNA.

The order Cornales consists of a diversity of plants from herbs to shrubs and trees. Taxonomic circumscription of Cornales and phylogenetic relationships within Cornales have long been controversial. Early in 1898, Harms recognized a broadly defined Cornaceae (*Alangium*, *Aucuba*, *Camptotheca*, *Cornus*, *Corokia*, *Curtisia*, *Davidia*, *Garrya*, *Griselinia*, *Helwingia*, *Kaliphora*, *Melanophylla*, *Nyssa*, and *Toricellia*) divided among seven subfamilies in the order Umbelliflorae. Many of the genera originally placed in Cornaceae by Harms (1898) were later treated as distinct families placed in Cornales or moved to other orders by various authors (Table 1). Several recent phylogenetic analyses using chloroplast DNA data suggested a Cornales clade that differs from all previous traditional concepts (Chase et al., 1993; Olmstead et al., 1993; Xiang et al., 1993, 1998; Xiang and Soltis, 1998; Xiang, 1999; Albach et al., 2001a, b). This new Cornales clade contains several genera that have been previously placed in Cornaceae (*Alangium*, *Camptotheca*, *Cornus*, *Curtisia*, *Davidia*, *Diplopanax*, *Mastixia*, *Nyssa*) and four other families (Grubbiaceae, Hydrangeaceae, Hydrostachyaceae, and Loasaceae). Nine genera (*Aralidium*, *Aucuba*, *Corokia*, *Garrya*, *Griselinia*, *Helwingia*, *Kaliphora*, *Melanophylla*, and *Toricellia*) previously placed in Cornaceae (or Cornales) by some authors were found to be related to noncornalean asterids in those studies. This molecular-based circumscription of Cornales was followed by the Angiosperm Phylogeny Group (APG) in 1998.

The placement of Hydrangeaceae and Loasaceae within the Cornales clade was found in all large-scale phylogenetic analyses using single or multiple molecular data sets (e.g., Downie and Palmer, 1992; Morgan and Soltis, 1993; Olmstead et al., 1993, 2000; Xiang, 1999; Soltis et al., 2000; Albach et al., 2001b). The two enigmatic monogeneric African families, Hydrostachyaceae and Grubbiaceae, first appeared in the cornalean clade in analyses of *rbcL* sequences for Loasaceae and Ebenales, respectively (Hempel et al., 1995; Morton et al., 1996). The affinity of these two families to Cornales was supported in further analyses for Cornales, asterids, eudicots, and angiosperms using chloroplast and nuclear genes (e.g., Xiang, 1999; Savolainen et al., 2000a, b; Soltis et al., 2000; Albach et al., 2001b; Xiang et al., 2002). Relationships within Cornales were not completely resolved in these previous studies, especially the placement of Hydrostachyaceae, for which there are few morphological features linking it to other cornalean taxa. Further, this family was always linked by an extremely long branch in the phylogenetic trees and its placement within Cornales was never strongly supported by bootstrap analyses (Hempel et al., 1995; Xiang, 1999; Soltis et al., 2000; Albach et al., 2001a; Xiang et al., 2002), and sometimes it was even placed outside of Cornales (Hufford et al., 2001). In the most recent analysis of *rbcL* and *matK* sequences for Cornales (Xiang et al., 2002), four major clades were identified: *Cornus*-*Alangium*, nyssoids-mastixioids (*Camptotheca*, *Davidia*, *Nyssa*, *Diplopanax*, and *Mastixia*), Hydrangeaceae (including *Hy-*

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TABLE 1. Comparison of taxonomic treatments of Cornales by different authors.

Harms (1898)	Wangerin (1910)	Hutchinson (1967)	Takhtajan (1980)	Cronquist (1981)
Umbelliflorae	Cornales	Araliales	Cornales	Cornales
Araliaceae	Alangiaceae	Alangiaceae	Alangiaceae	Alangiaceae
Umbelliferae	Cornaceae	Araliaceae	*Alangium*	Cornaceae
Cornaceae	Curtisioideae	*Helwingia*	Aucubaceae	*Aralidium*
Alangioideae	Cornoideae	Caprifoliaceae	Cornaceae	*Aucuba*
Alangium	*Aucuba*	Cornaceae	Cornoideae	*Cornus*
Curtisioideae	*Cornus*	*Afrocrania*	Curtisioideae	*Corokia*
Curtisia	*Corokia*	*Aucuba*	Mastixioideae	*Curtisia*
Cornoideae	*Griselinia*	*Chamaepericlymenum*	Davidiaceae	*Griselinia*
Aucuba	*Helwingia*	*Cornus*	*Davidia*	*Helwingia*
Cornus	*Kaliphora*	*Corokia*	Garryaceae	*Kaliphora*
Corokia	*Melanophylla*	*Curtisia*	Griseliniaceae	*Mastixia*
Griselinia	*Toricellia*	*Cynoxylon*	Helwingiaceae	*Melanophylla*
Helwingia	Mastixioideae	*Dendrobenthamia*	Melanophyllaceae	*Toricellia*
Kaliphora	*Mastixia*	*Griselinia*	*Melanophylla*	Garryaceae
Melanophylla	Garryaceae	*Kaliphora*	*Kaliphora*	Nyssaceae
Toricellia	Nyssaceae	*Mastixia*	Nyssaceae	*Camptotheca*
Davidioideae	Davidioideae	*Melanophylla*	*Camptotheca*	*Davidia*
Davidia	*Davidia*	*Swida*	*Nyssa*	*Nyssa*
Garryoideae	Nyssoideae	*Toricellia*	Toricelliaceae	
Mastixioideae	*Camptotheca*	Garryaceae		
Nyssoideae	*Nyssa*	Nyssaceae		
Camptotheca		*Camptotheca*		
Nyssa		*Davidia*		
		Nyssa		

drostachys)-Loasaceae, and *Curtisia-Grubbia*, with the first two clades being sisters, which in turn are sister to the third clade. However, these relationships and the placement of *Hydrostachys* were not strongly supported by bootstrap analyses.

In the present study, we collected a new molecular data set, nuclear 26S rDNA sequences, to further elucidate phylogenetic relationships within Cornales. The 26S rDNA sequences have been used to reconstruct phylogenetic relationships at various taxonomic levels of seed plants (e.g., Mishler et al., 1994; Ro et al., 1997, 1999; Kuzoff et al., 1998; Soltis and Soltis, 1998; Stefanovic et al., 1998; Ashworth, 2000; Chanderbali et al., 2001; Fan and Xiang, 2001; Fishbein et al., 2001; Neyland, 2001; Simmons et al., 2001; Soltis et al., 2001; Nickrent et al., 2002; Zanis et al., 2002). As 26S rDNA contains rapidly evolving expansion segments (ES) and conserved core (CC) regions (Clark et al., 1984; Dover and Flavell, 1984; Flavell, 1986), another goal of this study is to characterize the two regions (ES and CC) and evaluate their phylogenetic utilities in Cornales.

Long-branch attraction has long been recognized as a potential problem of parsimony analysis (Felsenstein, 1978; Swofford et al., 1996), whereas maximum likelihood (ML) methods incorporating appropriate substitution models may overcome this problem (Swofford et al., 1996). Given that *Hydrostachys* has been identified as having extremely long branches in all previous studies, we analyzed our data using parsimony and ML methods to see how the two methods perform differently regarding its placement.

MATERIALS AND METHODS

Sampling—Fifty-three taxa from Cornales were sampled for the 26S rDNA sequencing study, including *Alangium*, *Camptotheca*, *Cornus*, *Curtisia*, *Davidia*, *Diplopanax*, *Mastixia*, and *Nyssa*, two species of *Grubbia*, 12 genera from Hydrangeaceae, three genera of Loasaceae, and seven taxa of *Hydrostachys* representing six species. The sampling for *Alangium*, *Hydrostachys*, and *Mastixia* is broader than all the previous studies (e.g., Xiang et al., 1998, 2002), including more species from these genera in an attempt to break potential long branches. A broad range of taxa (15 species) from Ericales and euasterids were chosen as outgroups: five species were from Ericales (*Fouquieria columnaris*, Fouquieriaceae; *Halesia diptera*, Haesiaceae; *Sarracenia*, Sarraceniaceae; *Shortia galacifolia*, Diapensiaceae; *Styrax japonicus*, Styracaceae); four species were from euasterids I (one from Garryales [*Garrya elliptica*, Garryaceae], two from Lamiales [*Myoporum mauritianum*, Scrophulariaceae; *Veronica anagallis-aquatica*, Veronicaceae], one from Solanales [*Solanum lycopersicum*, Solanaceae]); and six species were from euasterids II (three from Apiales [*Apium graveolens*, Apiaceae; *Panax quinquefolius*, Araliaceae; *Petroselium crispum*, Apiaceae], two from Asterales [*Corokia cotoneaster*, Argophyllaceae; *Tragopogon dubius*, Asteraceae], and one from Aquifoliales [*Ilex opaca*, Aquifoliaceae]). The 26S rDNA sequences for all outgroups were downloaded from GenBank except those for *Sarracenia* and *Shortia*, whose sequences were generated in this study. A complete list of taxa, voucher, and GenBank accession numbers is available as Supplemental Data accompanying the online version of this article.

DNA extraction—Most genomic DNAs used in this study were isolated for previous *rbcL* and *matK* sequencing studies. The new DNAs were extracted from dried leaves of *Alangium chinense*, *Alangium kurzii*, *Cornus disciflora*, *Hydrostachys polymorpha*, *Hydrostachys* spp., *Mentzelia decapetala*, *Mastixia eugenioides*, *Mastixia pentandra* subsp. *chinensis*, *Petalonyx parryi*, and *Shortia galacifolia* using the modified cetyltrimethyl ammonium bromide (CTAB) method of Cullings (1992) with modifications described in Xiang et al. (1998).

Gene amplification—The entire 26S rDNA (approximately 3.3 kilobases [kb]) was successfully amplified from total DNA aliquots via a single polymerase chain reaction (PCR) run for a few taxa using the forward primer N-nc26S1 (5′-

TABLE 1. Extended.

Dahlgren (1983)	Takahtajan (1997)	APG (1998)	Thorne (2000)
Cornales	Cornales	Cornales	Cornales
Adoxaceae, Alangiaceae	Alangiaceae	Cornaceae	Vitineae
Alseuosmiaceae	*Alangium*	*Alangium*	Vitaceae
Anisophylleaceae	Cornaceae	*Camptotheca*	Vitoideae
Aquifoliaceae	*Afrocrania*	*Cornus*	Leeoideae
Aralidiaceae	*Cornus*	*Curtisia*	Gunnerineae
Aucubaceae	*Cynoxylon*	*Davidia*	Gunneraceae
Caprifoliaceae	*Swida*	*Diplopanax*	*Gunnera*
Cardiopteridaceae	Curtisiaceae	*Mastixia*	Cornineae
Columelliaceae	*Curtisia*	*Nyssa*	Cornaceae
Cornaceae, Davidiaceae	Davidiaceae	Grubbiaceae	*Cornus*
Dulongiaceae	*Davidia*	Hydrangeaceae	Nyssaceae
Eremosynaceae	Mastixiaceae	Hydrostachyaceae	Davidioideae
Escalloniaceae	*Diplopanax*	Loasaceae	*Davidia*
Garryaceae	*Mastixia*		Nyssoideae
Helwingiaceae	Nyssaceae		*Camptotheca*
Hydrangeaceae	*Camptotheca*		*Nyssa*
Icacinaceae	*Nyssa*		Mastixioideae
Montiniaceae, Nyssaceae			*Diplopanax*
Paracryphiaceae			*Mastixia*
Phellinaceae			Curtisiaceae
Pterostemonaceae			*Curtisia*
Sambucaceae			Alangiaceae
Sphenostemonaceae			*Alangium*
Stylidiaceae			
Symplocaceae			
Tetracarpaeaceae			
Torricelliaceae			
Tribelaceae, Viburnaceae			

CGACCCCAGGTCAGGCG-3′) and the reverse primer 3331rev (5′-ATCTCAGTGGATCGTGGCAG-3′) following Kuzoff et al. (1998) with slight modifications. For most species, the entire 26S rDNA sequence was amplified in two segments using primers N-nc26S1 with 1449rev (5′-ACCCATGTGCAAGTGCCGTT-3′) and N-nc26S5 (5′-CGTGCAAATCGTTCGTCT-3′) or N-nc26S6 (5′-TGGTAAGCAGAACTGGCG-3′) with 3331rev. Our PCR reactions are described in Fan and Xiang (2001).

Sequencing—The double-stranded (DS) PCR products were cleaned using 20% polyethylene glycol (PEG) 8000/2.5 mol/L NaCl (Morgan and Soltis, 1993; Soltis and Soltis, 1997). The purified DS DNA products were used as the templates for sequencing using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). Cycle-sequencing reactions (10 μL) were prepared by combining 2 μL terminator ready reaction mix, 2 μL sequencing buffer (200 mmol/L Tris-ph8.0, 5 mmol/L $MgCl_2$), 0.6 μL primer (5 μmol/L), 0.5 μL of 200 ng/μL cleaned PCR product, 0.5 μL dimethyl sulfoxide (DMSO), and 4.4 μL deionized water. Addition of 0.5 μL DMSO to the sequencing reactions resulted in cleaner sequences. Sixteen sequencing primers (N-nc26S1, N-nc26S3, N-nc26S4, N-nc26S5, N-nc26S6, N-nc26S8, N-nc26S10, N-nc26S12, N-nc26S14, 268rev, 641rev, 950rev, 1449rev, 2134rev, 2782rev, and 3331rev), described in Kuzoff et al. (1998), were used in different combinations to obtain the complete sequence of 26S rDNA. Cycle-sequencing was conducted on a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, Massachusetts USA) as follows: 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min.

Products of cycle-sequencing were cleaned using ethanol/sodium acetate precipitation (ABI Applied Biosystems, Foster City, California, USA) with an additional 95% ethanol wash. The cleaned sequencing products were analyzed on an ABI-377 automated sequencer (Applied Biosystems). The sequence chromatogram output files for all samples were checked and edited base by base manually before being aligned. For a few taxa (*Cornus controversa*, *Cornus sessilis*, *Curtisia*, and *Hydrostachys*), the above sequence primers did not yield complete sequences due to sequence divergence in some primer regions. Four new primers, 1227F (5′-GAACCCACAAAGGGTGTTGGTCG-3′) and 1793R (5′-CGCGACGTGCGGTGCTCTTCCAG-3′) for *C. controversa* and *C. sessilis* 1951F (5′-TTCGGGAAAAGGATTGGCTCTGAGG-3′) and 2857R (5′-GTGGTAACTTTTCTGACACCTCTAG-3′) for *Curtisia* and *Hydrostachys* were designed to solve this problem.

Parsimony analysis—The 26S rDNA sequences were initially aligned using ClustalX (Thompson et al., 1997) and then adjusted manually. The aligned sequences consist of 68 taxa and 3430 base pairs (bp) with small gaps (1–10 bp). The ES and CC regions of 26S rDNA were identified and located according to the coordinates for the expansion segments in the sequences of *Oryza sativa* (see Kuzoff et al., 1998) and *Cornus* (Fan and Xiang, 2001). The data matrix was analyzed with both parsimony and ML methods using PAUP* 4.0b10 (Swofford, 2002). For parsimony analysis, gaps were coded as missing data. Heuristic searches were performed using the MULPARS option with characters equally weighted, character states unordered, random taxon addition with 1000 replicates, and tree-bisection-reconnection (TBR) branch-swapping. To evaluate clade support, 10 000 replicates of bootstrap analysis (Felsenstein, 1985) were performed using fast heuristic search and TBR branch-swapping. In addition to analyses of the entire 26S rDNA sequences, ES and CC regions were also analyzed separately using parsimony to compare the relative phylogenetic utilities of the two regions.

Modeltest and maximum likelihood analysis—In order to find the appropriate substitution models for ML analyses of the 26S rDNA sequence data, *matK-rbcL* sequence data, and the combined 26S rDNA-*matK-rbcL* data, model searching was performed using the software Modeltest (Posada and Crandall, 1998). The ML analyses were subsequently conducted using the best model identified and parameter values estimated from Modeltest. For all ML analyses, heuristic searches were conducted using random taxon addition with 10 replicates. Due to the enormous amount of time required for bootstrap analyses of these large data sets (26S rDNA, 3430 bp; 26S rDNA-*matK-rbcL*, 6348 bp) using ML methods, we used neighbor-joining bootstrap analysis employing ML distance to approximate the bootstrap supports for the ML

trees. The same substitution model and parameters used in the ML analysis were used in the ML distance estimation. Ten thousand bootstrap replicates were conducted.

Incongruence test and combined data analysis—A combined data matrix of 26S rDNA-*matK*-*rbcL* including 42 taxa with sequences available for at least two of the three genes was constructed for a total evidence analysis. This matrix contains one species from Grubbiaceae, two from Hydrostachyaceae, four from Loasaceae, 13 from Hydrangeaceae, all 14 traditional cornalean genera, and eight outgroups. The aligned sequences contain a total of 6348 bp for each taxon, among which 3407 bp were from 26S rDNA, 1504 bp from *rbcL*, and 1437 bp from *matK*. An incongruence length difference test (ILD; Mickevich and Farris, 1981; Farris et al., 1994) was performed to assess the congruence between 26S rDNA and *matK*-*rbcL* sequence data. The ILD tests were conducted using the partition homogeneity test on PAUP* following Mason-Gamer and Kellogg (1996). One thousand homogeneity test replicates were conducted using heuristic search with 100 random taxon additions and TBR branch-swapping for each homogeneity replicate. Because an initial test suggested incongruence between the two data sets, further ILD tests for individual clades or excluding some clades from the matrix were conducted to identify lineages responsible for the incongruence. Both parsimony and ML analyses for combined data were conducted as described above.

RESULTS

Sequence data—The 26S rDNA sequences generated for the 68 species of Cornales and outgroups varied from 3340 to 3390 bp in length. The aligned matrix contained a total of 3430 bp, with small gaps between outgroups and Cornales taxa. Two additional insertions (bases 2096–2106 and 3251–3257) were detected in *Petalonyx*. Sequences for most species were complete except those for *Decumaria* and one species of *Grubbia* (*G. rosmarinifolia*), which were missing approximately half of the 3′ end. The sequences for *Hydrostachys angustisecta*-HS-4, *H. insignis*, and *H. imbricata* were also incomplete with a portion of the 5′ end missing. Including or excluding these taxa with incomplete sequences in the analyses did not affect the placements of remaining taxa in the resulting trees, thus we included them in the analyses for a broader sampling. Among the 68 sequences of Cornales and outgroups, 1048 of the 3430 sites are variable (30.56%) and 557 sites (16.24%) are parsimony informative.

Twelve expansion segments (ES) were identified in the 26S rDNA sequence data matrix including outgroups. The expansion segments span a total of 1052 bp, of which 580 sites (55.13%) are variable and 393 sites (37.36%) are phylogenetically informative. These values are approximately 3–5 times higher than from core conserved (CC) regions, which contain 2378 bp, of which 468 sites (19.68%) are variable and only 164 sites (6.90%) are phylogenetically informative.

Among the 42 sequences of the combined data set (26S rDNA, *matK*, and *rbcL*), 2022 of the 6348 sites (31.85%) are variable and 1168 sites (18.40%) are phylogenetically informative. Among the 1168 phylogenetically informative sites, there are 466 from 26S rDNA, 454 from *matK*, and 248 from *rbcL*.

Phylogenetic relationships based on 26S rDNA sequences—Parsimony analysis of 26S rDNA sequences alone found 47 most parsimonious trees of 2947 steps (Fig. 1). Eight major clades (supported by bootstrap support values of over 65%) were identified in all parsimonious trees: (1) *Cornus*; (2) *Alangium*; (3) nyssoids (*Nyssa*, *Davidia*, and *Camptotheca*); (4) mastixioids (*Diplopanax* and *Mastixia*); (5) *Curtisia*-*Grubbia*; (6) Loasaceae; (7) Hydrangeaceae; and (8) *Hydrostachys* (Fig. 1). The relationships among these major clades suggested in the strict consensus tree are shown in Fig. 1. None of the nodes connecting the major clades is supported by bootstrap analysis values of greater than 50% (Figs. 1 and 2). However, the differences among the 47 trees mostly involved only arrangements within Hydrangeaceae and among outgroup taxa. Compared to previous *matK*-*rbcL*-based phylogeny, the strongly supported *Cornus*-*Alangium* clade is interrupted by *Hydrostachys*, which is placed as the sister of *Cornus* in the 26S rDNA trees (9% bootstrap value, Figs. 1 and 2); the monophyly of Hydrangeaceae-Loasaceae is also contradicted by the 26S rDNA strict consensus trees.

Modeltest indicated GTR + I + Γ is the best-fit model for the 26S rDNA sequence data. This GTR + I + Γ model incorporates both unequal base frequencies and different rates for all six substitutions and allows for among-site variation of substitution rates. A single best tree was found from the ML analysis using the GTR + I + Γ model and parameter values estimated from the model test. The same eight major clades as those found in the parsimony analysis were identified in the ML tree (Fig. 3), but the arrangements among these clades were different between the parsimony and ML trees. The monophyly of *Cornus*-*Alangium* was recovered, although without high bootstrap support (28%). The placement of *Hydrostachys* is dramatically different between the parsimony and ML trees. It is placed as the sister of *Cornus* in the parsimony analysis, whereas in the ML analysis it is placed as the sister of Loasaceae (Figs. 1 and 3). In both cases, this genus is monophyletic and connected by a long branch.

Cornus forms a monophyletic group, with four subclades: *C. canadensis*-*C. suecica*-*C. unalaschkensis* (the dwarf dogwoods); *C. mas*-*C. officinalis*-*C. sessilis* (the cornelian cherries); *C. florida*-*C. kousa*-*C. disciflora* (the big-bracted dogwoods); and *C. oblonga*-*C. racemosa*-*C. controversa*-*C. walteri* (the blue- or white-fruited group). Species of *Mastixia* also form a strongly supported monophyletic group in the mastixioids clade (bootstrap support [BS] = 100%), which is sister to *Diplopanax* with high bootstrap support (83% and 97%). *Nyssa* is monophyletic (98%, 100%), and *Camptotheca* and *Davidia* are sisters (57%, 60%) in the nyssoids clade. *Petalonyx* is sister to *Mentzelia* among the three sampled genera of Loasaceae. In Hydrangeaceae, subclade Hydrangeeae, consisting of *Platycrater*, *Decumaria*, *Pileostegia*, *Schizophragma*, *Hydrangea*, *Broussaisia*, and *Cardiandra*, and subclade Philadelpheae, consisting of *Deutzia*, *Fendlerella*, and *Philadelphus*, were recognized and well supported. The monophyly of Jamesioideae, however, was not supported in either parsimony or ML trees (Figs. 1–3). Within Hydrostachyaceae, three species from Madagascar (*H. angustisecta*, *H. imbricata*, and *H. multifida*) form a basal clade and are relative to the three Malawi species (*H. polymorpha*, *H. insignis*, and *H. augustisecta*) and one unidentified species from Madagascar (Figs. 1–3).

The analysis of ES regions using parsimony-generated trees with topologies similar to those derived from the entire sequences (trees not shown). For example, the same eight major clades were similarly identified in the ES trees, and *Hydrostachys* was placed within Cornales. However, the ES trees have less resolution within and among major clades and lower bootstrap support for major clades than trees inferred from the entire sequences. The analysis of CC regions alone produced over 10 000 trees without finishing searching, showing unex-

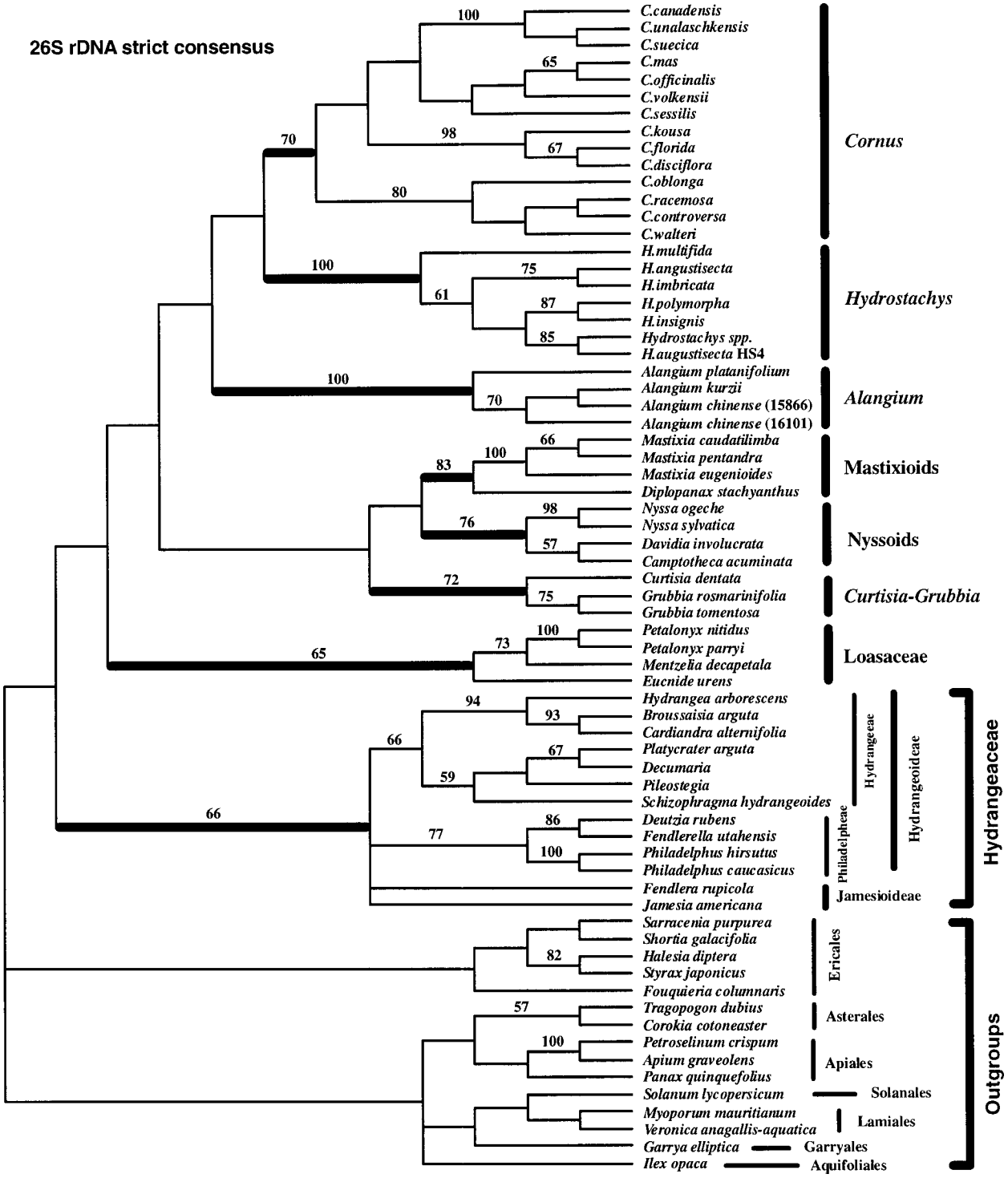

Fig. 1. The strict consensus tree from parsimony analysis of 26S rDNA sequences. Bootstrap values (>50%) are indicated above branches. The major clades are marked by thickened lines.

pected relationships within Cornales in the strict consensus tree, such as the collapse of strongly supported clades, including the *Cornus* clade (*C. volkensii* was separated from the other *Cornus* species, and placed in the outgroup), the nyssoids clade, and the Loasaceae clade (*Mentzelia* was placed as the most basal lineage of Cornales).

Incongruence test—The phylogenetic trees of Cornales inferred from 26S rDNA sequences were substantially different from those based on *matK* and *rbcL* sequences regarding the relationships among the major clades and within Hydrangeaceae (Xiang et al., 2002; also compare Figs. 3 and 4). Although the discrepancy mainly involved deep nodes that are

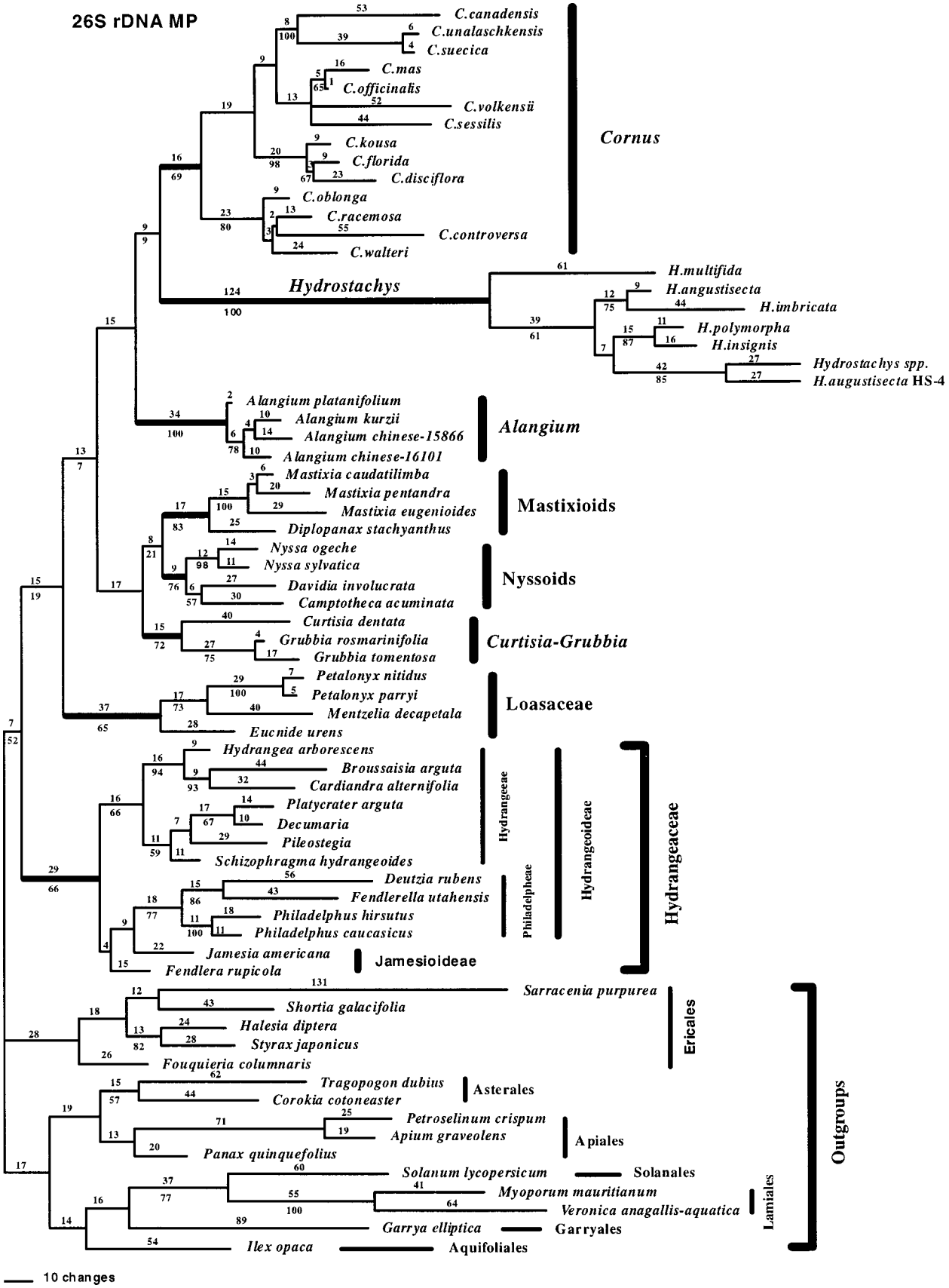

Fig. 2. One of 47 equally parsimonious trees from parsimony analysis of 26S rDNA sequences (tree length = 2947 steps, CI = 0.487 excluding uninformative characters, RI = 0.632). Base substitutions are indicated above branches; bootstrap values (>5%) are indicated below branches. The major clades are marked by thickened lines.

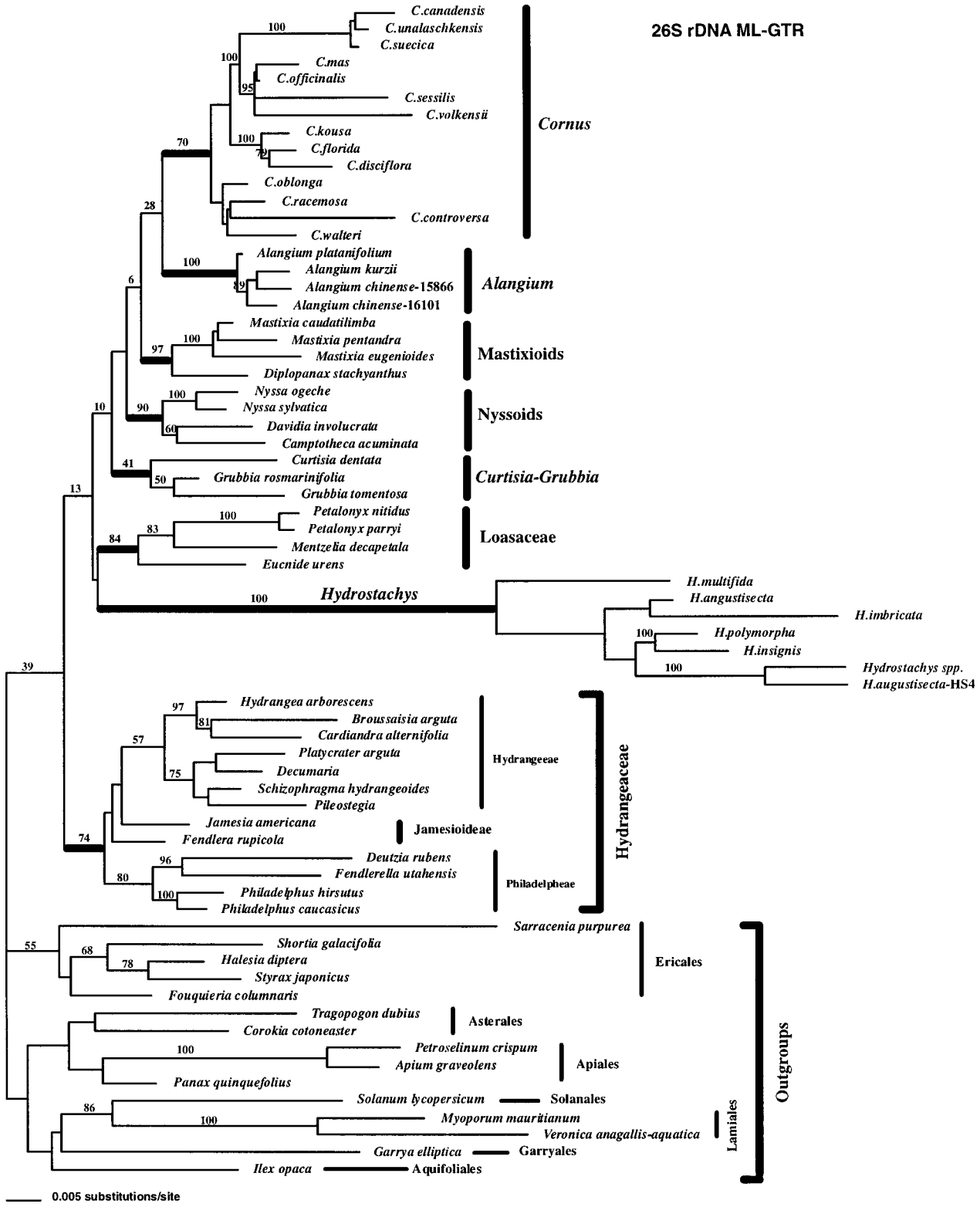

Fig. 3. The maximum likelihood tree from analysis of the 26S rDNA sequences using the GTR + I + Γ model with the following parameter values: rate matrix of R with AC = 1.094, AG = 2.342, AT = 1.599, CG = 1.103, CT = 7.888; base frequencies = A, 0.235; C, 0.247; G, 0.314; T, 0.203; proportion of invariable sites = 0.488; α of gamma distribution = 0.522. Bootstrap values (>5%) obtained using neighbor-joining bootstrap analysis that employed ML distance (see Materials and Methods) are indicated above branches (−Ln likelihood = 21 451.620). The major clades are marked by thickened lines.

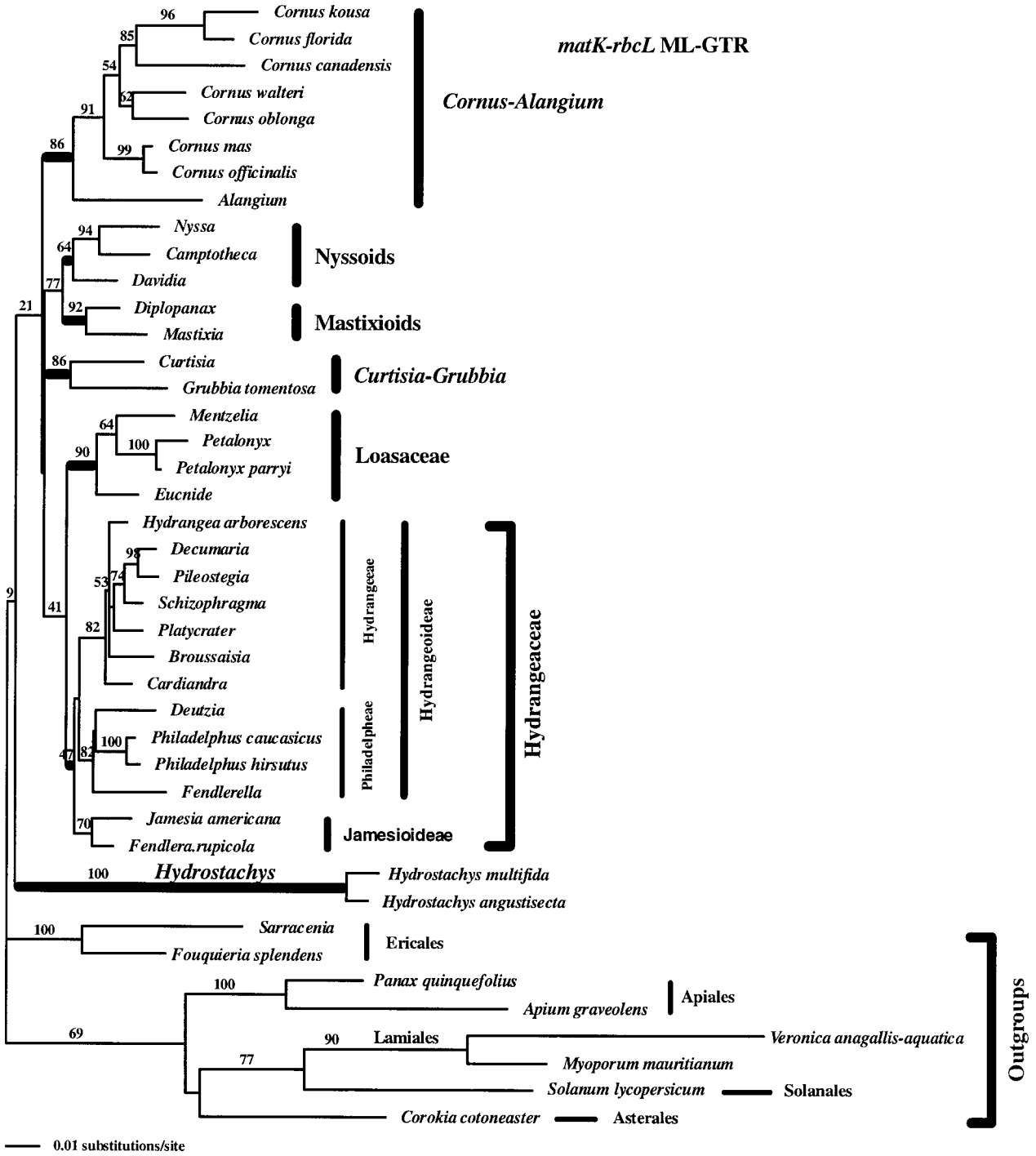

Fig. 4. The maximum likelihood tree from analysis of combined *matK* and *rbcL* sequences of 42 taxa including 26S rDNA sequences that use GTR + I + Γ model with these parameter values: rate matrix of R with AC = 1.389, AG = 2.274, AT = 0.393, CG = 0.919, CT = 2.274; base frequencies = A, 0.296; C, 0.187; G, 0.189; T, 0.328; proportion of invariable sites = 0.384; α of gamma distribution = 1.256. The ML bootstrap values (>5%) are indicated above branches (−Ln likelihood = 17 850.031). The major clades are marked by thickened lines.

mostly weakly supported in both cpDNA trees and 26S rDNA trees, we performed ILD tests to evaluate the congruence of the two data sets. The results indicated significant incongruence between the *matK-rbcL* and 26S rDNA sequence data ($P = 0.001$). Subsequent successive ILD tests excluding individual major lineage one at a time were further conducted to locate the problematic lineages. Results revealed that much of the incongruence was attributed to a single group, Hydrangeaceae. The P value of ILD tests increased ($P = 0.003$) only when Hydrangeaceae and outgroups were excluded (Table 2). Further, ILD tests were performed for each major lineage and results also showed that Hydrangeaceae, in particular the subclade Hydrangeeae, is the only ingroup showing significant disagreement between the two data sets (Table 2).

TABLE 2. Incongruence length difference (ILD) test between 26S rDNA and *matK-rbcL* data sets with different major lineages excluded and included alone.

Lineages excluded/included alone	Sum of tree lengths for original partition (excluded/included alone)	*P* (excluded/included alone)
Cornus-Alangium	4018/589	0.001**/0.121
Nyssoids-mastixioids	4215/401	0.001**/0.08
Loasaceae	4335/NA	0.001**/NA
Hydrangeaceae	3804/815	0.003**/0.001**
Hydrangeeae	4088/359	0.001/0.001**
Curtisia-Grubbia	4411/NA	0.001**/NA
Curtisia-Grubbia-Loasaceae	4144/542	0.001**/1.0
Hydrostachyaceae	4223/NA	0.001**/NA
Hydrostachyaceae + outgroups	2503/2054	0.001**/0.01**
Outgroups	2965/1594	0.001**/0.22

** Significant discordances between partitions of 26S rDNA and *matK-rbcL*. NA = not applicable, because Modeltest required at least four taxa.

Phylogenetic relationships based on combined 26S rDNA, matK, and rbcL sequences—Considering that the topological discrepancy between 26S rDNA and *matK-rbcL* trees mainly involved weakly supported nodes, and potentially only a single lineage exhibits conflicts between the two data sets, we performed analyses of the combined 26S rDNA and *matK-rbcL* sequences. A single most parsimonious tree was found from the parsimony analysis of combined data (tree length 4735, consistency index [CI] = 0.570, retention index [RI] = 0.547) (Fig. 5). The tree shows the monophyly of *Cornus-Alangium*, Loasaceae-Hydrangeaceae (weakly supported), nyssoids-mastixioids, and *Grubbia-Curtisia*. *Hydrostachys* groups with outgroups, sister to the remainder of Cornales (Fig. 5). A model test similarly suggested that the GTR + I + Γ model best fits the combined data. The ML analysis using this model resulted in a single tree (Fig. 6) with topology showing the same eight major clades and relationships within and among the clades similar to those in the *matK-rbcL* tree (Fig. 4). *Hydrostachys* was placed at the base of Cornales with low bootstrap supports (Fig. 6). However, bootstrap and CI values increased significantly for most clades in the combined 26S rDNA-*matK-rbcL* trees (compare Figs. 2 and 3 with 5 and 6).

DISCUSSION

Differential phylogenetic utility of the ES and CC regions of 26S rDNA in Cornales—The large subunit of rDNA is structured as a mosaic of core conserved and variable domains (as defined as expansion segments by Clark et al. [1984]). The ''core'' conserved segments have primary and secondary structures conserved in prokaryotes and eukaryotes (Hancock and Dover, 1990). The expansion segments are responsible for the difference in size between eukaryotic and prokaryotic rDNA, and they evolve much faster than conserved core regions. Despite their rapid evolution, expansion segments still contain a conserved secondary structure and show nucleotide composition constraints in some species (Hassouna et al., 1984; Gorab et al., 1995). Comparative analyses confirmed that the base substitution rates in the expansion segments of 26S rDNA are lower than those observed in nuclear noncoding regions or neutral bases (Larson and Wilson, 1989). Compared to animals, the considerable length mutation of the expansion segments observed in animal rDNA is not found in angiosperms (Kolosha and Fodor, 1990), and consequently, the expansion segments may be more alignable among angiosperms and the point mutations in the sequence may be phylogenetically informative at different taxonomic levels in plants (Kuzoff at al., 1998). Because of the relatively high rate of substitutions in the expansion segments, Larson (1991) suggested that the expansion regions should be excluded from phylogenetic analyses of taxa with a common ancestor older than 200 million years ago (MYA) due to possible saturation of substitutions. The 26S rDNA in Cornales contains 12 expansion segments, which evolve about 3–5 times as fast as the conserved core regions. This ratio is much lower than that observed for other angiosperms (6.4 to 10.2 times; Kuzoff et al., 1998). The analyses of separate partitions of ES and CC regions using parsimony suggested that in Cornales most of the phylogenetic signals of 26S rDNA are from the ES regions and the CC regions alone are not sufficient to resolve meaningful relationships in the group, although it might be at higher taxonomic levels.

Discrepancies between parsimony and maximum likelihood analyses—It is well recognized that one potential problem of parsimony analysis is the inconsistency of the method if substitution rates are high and unequal among lineages (Felsenstein, 1978; Swofford et al., 1996, 2001). In this case, unrelated taxa with high rates (shown as long branches in the data matrix) will be likely attracted to each other in a simple parsimony analysis. An ML analysis implementing appropriate substitution model(s) is supposed to be able to largely overcome this long branch problem (Felsenstein, 1981; Swofford et al., 1996). Our analyses of 26S rDNA sequences and combined 26S rDNA-*matK-rbcL* sequences using parsimony and ML methods suggested different placement for the long-branched *Hydrostachys*. Parsimony analysis of 26S rDNA sequence data placed *Hydrostachys* in the *Cornus-Alangium* clade, whereas the ML analysis of 26S rDNA data placed it with the Loasaceae (Figs. 1–3). Analyses of the combined 26S rDNA-*matK-rbcL* using parsimony-grouped *Hydrostachys* with outgroups, whereas ML analysis of the combined data grouped it with Cornales and placed it as the sister to the remainder of the Cornales clade. The placements of *Hydrostachys* in the 26S rDNA and combined data are both weakly supported. Additional discrepancies of relationships among major lineages between parsimony and ML analyses were also found in our analyses. For example, the sister relationship between *Cornus* and *Alangium* identified in the ML analysis was congruent with all previous chloroplast data analyses and supported by morphological characters (Eyde, 1988). Neverthe-

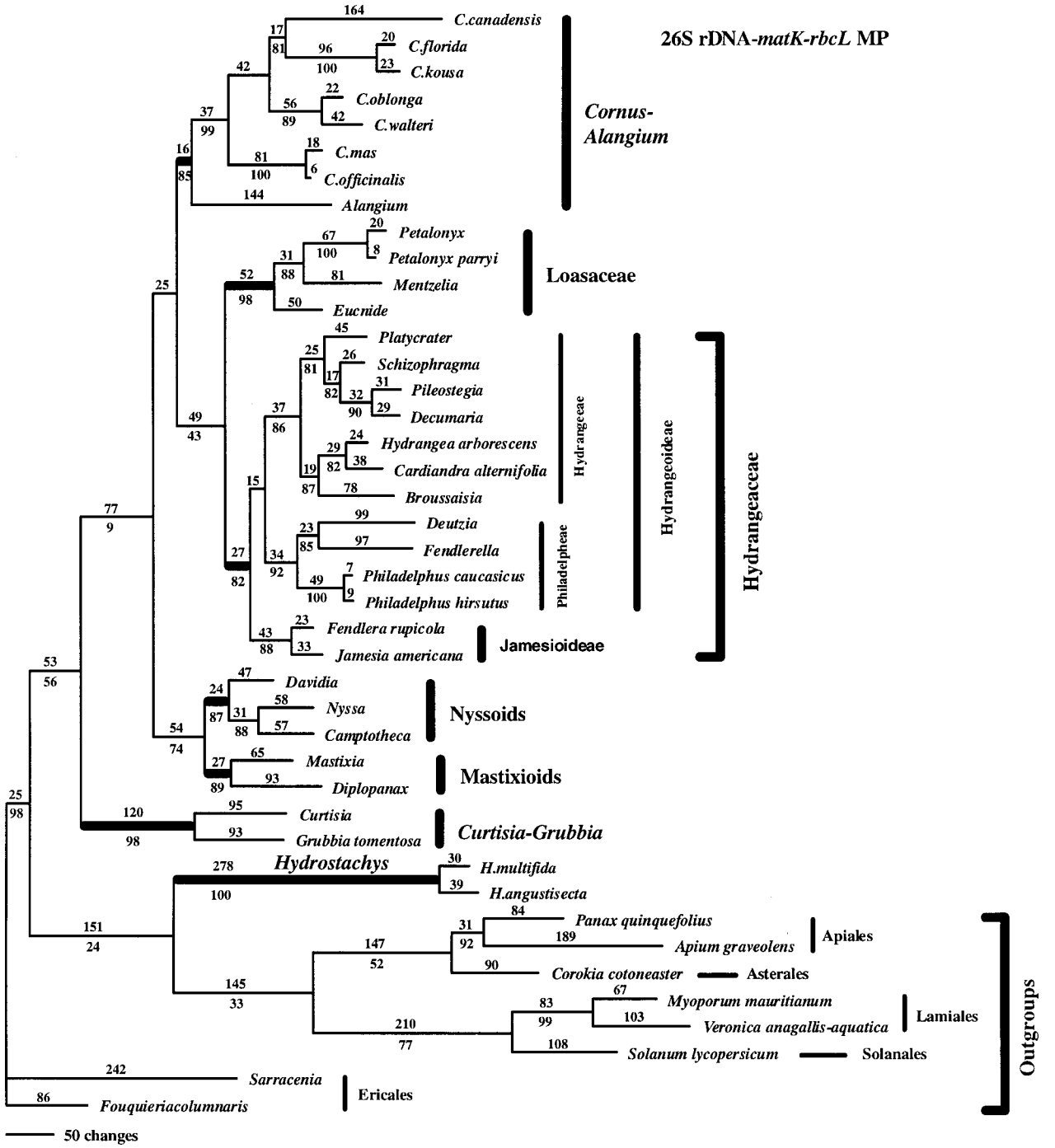

Fig. 5. The single most parsimonious tree from analysis of combined 26S rDNA-*matK*-*rbcL* sequence data (tree length = 4735 steps, CI = 0.570 excluding uninformative characters, RI = 0.547). Base substitutions are indicated above branches; bootstrap values (>5%) are indicated below branches. The major clades are marked by thickened lines.

less, this relationship was broken off by *Hydrostachys* in 26S rDNA parsimony analysis. The monophyly of traditional cornalean taxa including *Alangium*, *Cornus*, nyssoids, and mastixioids recognized in the ML trees (Fig. 3) are in agreement with morphology and previous chloroplast data analysis, but the monophyly of these taxa was not identified in the parsimony trees (Figs. 1, 2, and 5). However, it must be noted that these relationships showing discrepancies are generally not strongly supported in either parsimony or ML trees.

Placements of Hydrostachyaceae and the long branch—The systematic affinity of the African aquatic family Hydrostachyaceae (consisting of only *Hydrostachys* with 22–25 species) has long been controversial. It has been placed near Podostemaceae (Bentham and Hooker, 1880) or as the distinct order Hydrostachyales allied with Lamiales and Scrophulariales (Takhtajan, 1969, 1980, 1997; Dahlgren, 1980, 1983, 1989; Leins and Erbar, 1988, 1990; Wagenitz, 1992) and in Bruniales (Thorne, 1968, 1983, 1992, 2000) and Callitrichales

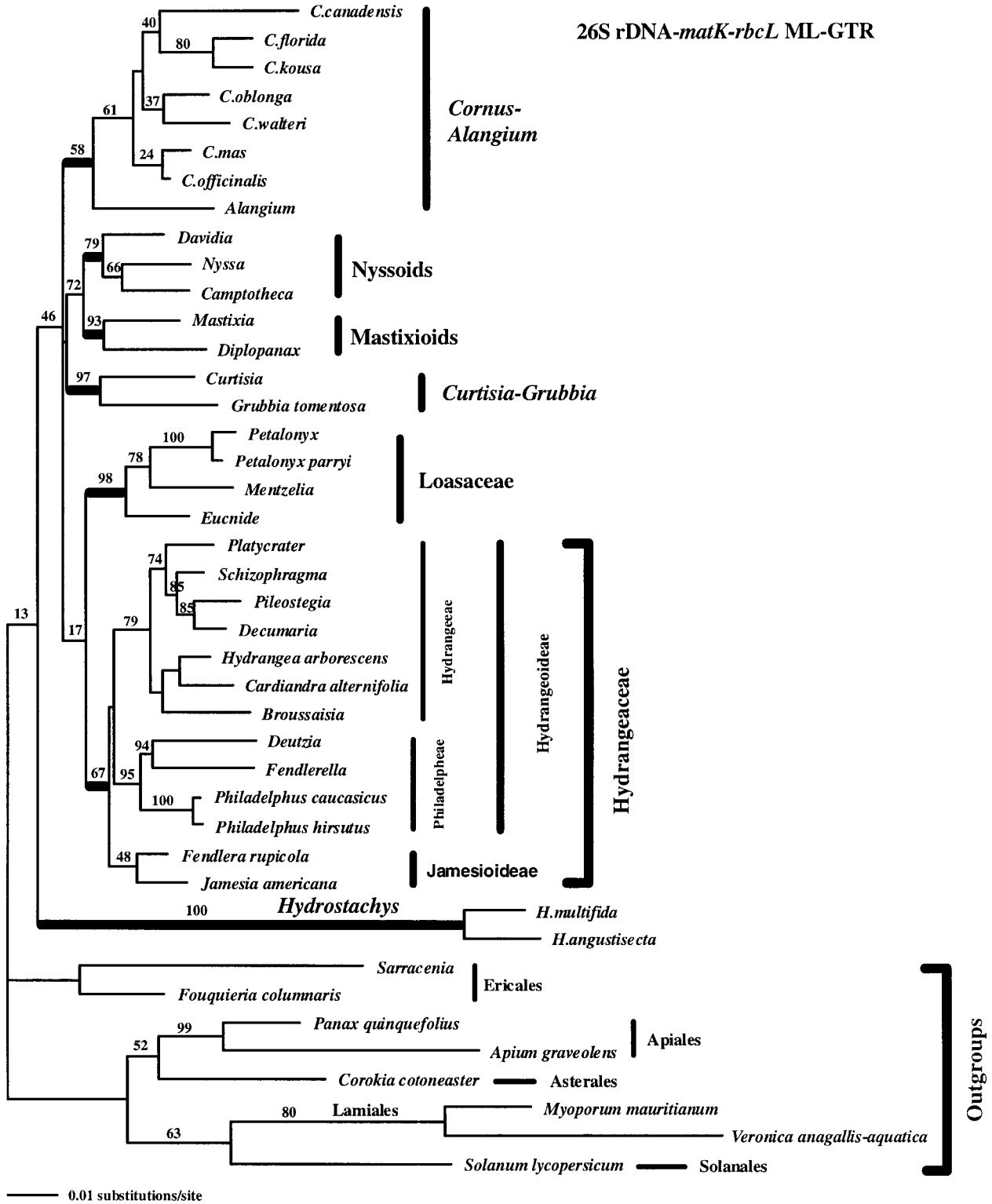

Fig. 6. The maximum likelihood tree from analysis of combined 26S rDNA-*matK-rbcL* sequence data using the GTR + I + Γ model with parameter values: rate matrix of R with AC = 1.279, AG = 2.048, AT = 0.739, CG = 0.958, CT = 4.287; base frequencies = A, 0.265; C, 0.219; G, 0.257; T, 0.258; proportion of invariable sites = 0.498; α of gamma distribution = 0.735. Bootstrap values (>5%) approximated using neighbor joining employed with ML distance (see Materials and Methods) are indicated above branches (−Ln likelihood = 34716.487). The major clades are marked by thickened lines.

(Cronquist, 1981) based on the features in morphology. The family was first linked to Cornales in the *rbcL* sequence analysis of Loasaceae by Hempel et al. (1995). More recent molecular data analyses (Xiang, 1999; Albach et al., 2001a, b; Xiang et al., 2002) and evidence from phytochemistry (Rønsted et al., 2002) further supported the placement of this family in Cornales. A majority of previous phylogenetic analyses suggested a position of *Hydrostachys* within Hydrangeaceae, with low bootstrap support (e.g., Hempel et al., 1995; Xiang, 1999; Xiang et al., 2002). A few possible synapomorphies of Hydrangeaceae and *Hydrostachys* were identified by previous authors, such as two or more free styles, capsules, and numerous, anatropous ovules per locule (see Xiang, 1999; Albach et al., 2001a). The ML analyses of 26S rDNA sequence data and combined 26S rDNA-*matK*-*rbcL* sequence data, as well as parsimony analysis of 26S rDNA data, all suggested that *Hydrostachys* is a member of Cornales but revealed new placements in Cornales different from those suggested in previous analyses. For example, the ML tree of 26S rDNA placed *Hydrostachys* as sister to Loasaceae (Fig. 3). The ML tree of the combined 26S rDNA-*matK*-*rbcL* shows that *Hydrostachys* is sister to the remainder of Cornales (Fig. 6). The placement of *Hydrostachys* with outgroups in the parsimony analysis of the combined data is likely a result of long-branch attraction, given that the branches leading to *Hydrostachys* and the outgroup clade are both long (Fig. 5).

As discussed above, long-branch attraction is a concern in phylogenetic analyses using a parsimony approach (Felsenstein, 1978; Swofford et al., 2001). Both simulation (e.g., Hillis and Huelsenbeck, 1993; Huelsenbeck, 1995; Yang, 1996; Siddall, 1998; Pol and Siddall, 2001) and empirical studies (e.g., Omilian and Taylor, 2001; Litvaitis, 2002) have shown that long-branch attraction can result in wrong phylogenies when using a parsimony method. One recommended solution to long-branch attraction is to increase the sampling of long-branched taxa to decrease the branch length. In our 26S rDNA analysis, seven species of *Hydrostachys* were sampled with the attempt to reduce the long branch of the genus revealed in previous various cpDNA analyses (Xiang, 1999; Xiang et al., 2002). With increasing sampling, the branches leading to *Hydrostachys* in the parsimony and ML 26S rDNA trees were significantly reduced in length compared to those in the cpDNA trees with only one or two species sampled (Xiang, 1999; Albach et al., 2001a, b; Xiang et al., 2002). In the 26S rDNA trees, the branch of *Hydrostachys* is not much longer than the outgroup branches (Figs. 2 and 3) and only about twice as long as the longest ingroup branches (Figs. 2 and 3). In all phylogenetic analyses of cpDNA sequence data sampling a single or two species (e.g., Xiang, 1999; Xiang et al., 2002; Figs. 4–6), the branches of *Hydrostachys* were much longer, sometimes several times longer, than the longest branches of the ingroups, and much longer than the longest outgroup branches. These results demonstrated that increasing sampling of the long-branched group indeed substantially decreased the branch length.

Many studies have suggested that organisms that are highly modified may morphologically have accelerated rates of molecular evolution (Nickrent and Starr, 1994; DePamphilis et al., 1997; Les et al., 1997; Mallat and Sullivan, 1998; Soltis et al., 1999, 2000; Chase et al., 2000; Albach et al., 2001a). *Hydrostachys*, due to its aquatic habit, is morphologically highly divergent from the remaining cornalean taxa (e.g., pinnate compound leaves, tuber-like rhizomes, and dense spike inflorescence). Its long branches revealed in previous analyses could be viewed as evidence of its elevated rates of molecular evolution in the genus. However, long branches could be simply a result of the incomplete sampling from the genus, as increasing sampling substantially reduced the branch length in our 26S rDNA analysis. However, this sampling effect is less clear when examining the trees from combined nuclear and cpDNA sequences (Figs. 5 and 6). Based on the combined 26S rDNA-*matK*-*rbcL* sequence data, the separation of *Hydrostachys* from the rest of Cornales might have occurred very early, before the origin of all other cornalean major lineages (Fig. 6).

Relationships of Grubbia and Curtisia—Grubbiaceae, another monogeneric family of Cornales from southern Africa, in addition to Curtisiaceae and Hydrostachyaceae, represents another family difficult to place in the classification of flowering plants. Both separate and combined data analyses in the present study suggested that *Grubbia* and *Curtisia* are sisters, in agreement with the previous finding from the *matK*-*rbcL* data (Xiang et al., 2002). The sister relationship between *Grubbia* and *Curtisia* is supported by high bootstrap values in all analyses. Unlike *Hydrostachys*, which shows no apparent morphological similarities with other cornalean taxa, *Grubbia* and *Curtisia* share several morphological features that are common in the Cornales (see Xiang, 1999). Therefore, the finding of a close relationship between the two genera both endemic to southern Africa is not a surprise. The circumscription of Grubbiaceae including both *Grubbia* and *Curtisia* as proposed by Xiang et al. (2002) is strongly supported. Relationships of *Grubbia*-*Curtisia* to other cornalean taxa are not clearly resolved.

Monophyly of nyssoids, mastixioids, Cornus, and Alangium—The monophyly of nyssoids, mastixioids, *Cornus*, and *Alangium* was suggested in the ML analysis of 26S rDNA data (Fig. 3). This clade is also supported by a few nonmolecular characters (e.g., fleshy drupaceous fruit with germination valves on fruit stones, H-shaped thinning in pollen aperture, and the lack of central bundles in gynoecial vasculature), and largely corresponds to the Cornaceae of Eyde (1988). However, given the low bootstrap support for the clade (Fig. 3), it is better to maintain the nyssoids and mastixioids as separate families as discussed in Xiang et al. (2002).

The monophyly of the nyssoids, mastixioids, and *Cornus*-*Alangium* subclades is strongly supported in the combined 26S rDNA-*matK*-*rbcL* data analyses. The sister relationship between *Cornus* and *Alangium* has been also recognized in previous molecular studies (Xiang et al., 1993, 1998, 2002; Xiang, 1999) and is also supported by some morphological and embryological characters (e.g., unitegmic and crassinucellate ovules; degeneration of nucleus followed by the differentiation of an integumentary tapetum; single-celled archesporium; see Chopra and Kaur, 1965; Eyde, 1968, 1988). Based on this evidence, Xiang et al. (2002) proposed a Cornaceae consisting of *Cornus* and *Alangium* following Soltis et al. (2000). Because *Alangium* has long been recognized as a monogeneric family and the name Alangiaceae has been widely used, we proposed here to separate *Cornus* and *Alangium* in Cornaceae and Alangiaceae, respectively.

The relationships within the nyssoids vary between separate data partitions and combined data. In analysis of 26S rDNA sequences, *Camptotheca* is sister to *Davidia* (57%, 60%; Figs.

2 and 3), whereas in analyses of combined 26S rDNA-*matK*-*rbcL* sequence data, *Nyssa* is strongly supported to be the sister of *Camptotheca* (BS = 83%), and the two, in turn, are sister to *Davidia* (Figs. 5–6). These relationships were also found in earlier and present analyses of *matK* and *rbcL* sequences (Xiang et al., 1998, 2002). A closer relationship of *Camptotheca* to *Nyssa* is also supported by some nonmolecular data (e.g., the structure of the fruits and the inflorescences, Eyde, 1963, 1967; wood anatomy, Titman, 1949; palynology, Eramian, 1971 and Eyde and Barghoorn, 1963; fatty acids, Bate-Smith et al., 1975 and Hohn and Meinschein, 1976).

The sister relationship between *Mastixia* and *Diplopanax* (Fig. 6) was first recovered in the combined *rbcL*-*matK* sequence analysis of Xiang et al. (2002) and again recovered in the present study with high bootstrap support in all analyses. The close relationship between *Mastixia* and *Diplopanax* was earlier recognized by Eyde and Xiang (1990) and further supported by Zhu and Xiang (1999) via studies of fruit, leaf, and floral anatomic structures. Both genera produce flowers with hooked petals that are arranged in paniculate inflorescences, fruits that have a bony stone with an intrusive germination valve lacking a longitudinal septum, and a one-seeded chamber.

Phylogenetic relationships in Hydrangeaceae and Loasaceae—Two strongly supported monophyletic groups, which correspond to the two tribes Hydrangeeae and Philadelpheae, were recognized in Hydrangeaceae in both separate and combined analyses. The monophyly of Jamesioideae was not recognized in the 26S rDNA sequence analyses, but was in the tree based on combined data (Figs. 1–6). The relationships within Hydrangeeae suggested by 26S rDNA and *matK*-*rbcL* were different (Figs. 1–4). The combined 26S rDNA-*matK*-*rbcL* data agreed with the *matK*-*rbcL* data in placing *Pileostegia* + *Decumaria* as the sister of *Schizophragma* with high bootstrap support (Figs. 4, 5, and 6). The close relationships among the genera are also supported by morphological data (Hufford, 1992, 1997) and recovered in previous phylogenetic analyses (Soltis et al., 1995; Hufford et al., 2001; Xiang et al., 2002). Morphological data suggested that *Platycrater* was outside of the *Hydrangea* clade (including genera of *Hydrangea*, *Pileostegia*, *Decumaria*, *Broussaisia*, and *Schizophragma* in this study), a clade supported by a synapomorphic character of diplostemony (Hufford, 1997). However, all molecular analyses (Soltis et al., 1995; Xiang, 1999; Hufford et al., 2001; and the present study) placed *Platycrater* within the *Hydrangea* clade, suggesting that diplostemony might have been lost in *Platycrater*, as previously hypothesized by Hofford et al. (2001). The relative relationships among *Hydrangea*, *Broussaisia*, and *Cardiandra* are different in 26S rDNA and combined 26S rDNA-*matK*-*rbcL* trees all with strong bootstrap supports (Figs. 1–3, 5, and 6). However, these relationships were not revealed in previous phylogenetic analyses with a more thorough sampling of genera of Hydrangeaceae (Soltis et al., 1995; Hufford et al., 2001; Xiang et al., 2002). In those analyses with a complete sampling of genera in the family, *Cardiandra* and *Deinanthe* were recognized as sisters and placed at the base within the Hydrangeeae clade (Hufford et al., 2001; Xiang et al., 2002). Therefore, the sister relationships among these three taxa revealed in the present study is likely a result of incomplete sampling.

Only four species of Loasaceae representing two of the three subfamilies (Gronovioideae and Mentzelioideae) were sampled in this study, thus relationships within Loasaceae cannot be appropriately addressed with confidence. However, the two genera *Eucnide* and *Mentzelia*, from subfamily Mentzelioideae, do form a monophyletic group in the combined data analyses (bootstrap value 100%). The two are, in turn, sister to *Petalonyx* (from subfamily Gronovioideae). These relationships are also congruent with earlier studies using *rbcL* sequence data (Hempel et al., 1995) and our *matK*-*rbcL* sequence analysis (Fig. 3). However, 26S rDNA sequence data alone placed *Mentzelia* sister to *Petalonyx*, agreeing with the *matK* and ITS sequence data (Moody et al., 2001). *Eucnide* and *Mentzelia* share many morphological characters in floral structures (e.g., polystemonous, multicarpellate, multiovulate, and dehiscent fruits). However, some possible morphological synapomorphies (e.g., the absence of the petal-stamen plate in *Mentzelia* and Gronovioideae) may unite *Mentzelia* and Gronovioideae (including *Petalonyx*). This discrepancy may be due to either inadequate sampling of Loasaceae in different studies and/or different phylogenetic signals between data sets, which needs further investigation.

Conclusion—Phylogenetic analyses of nuclear DNA sequence data and combined nuclear and chloroplast DNA sequence data further support a Cornales consisting of *Cornus*, *Alangium*, nyssoids, mastixioids, Hydrangeaceae, Loasaceae, Grubbiaceae (*Grubbia*-*Curtisia*), and Hydrostachyaceae (*Hydrostachys*). Four most-inclusive major clades in Cornales (*Cornus*-*Alangium*, nyssoids-mastixioids, Hydrangeaceae-Loasaceae, and *Grubbia*-*Curtisia*) identified in previous *matK*-*rbcL* sequence analyses (Xiang et al., 1998, 2002; Xiang, 1999) were also recovered in analyses of the combined nuclear and chloroplast DNA sequence data in the present study. The combined 26S rDNA-*matK*-*rbcL* sequence data suggested that Hydrostachyaceae probably branched early from the remainder of Cornales. Relationships among major lineages of Cornales are weakly supported by bootstrap analyses, similar to previous studies. This uncertainty of relationships among major lineages of Cornales, despite rigorous analyses of a large number of characters, may reflect an early rapid radiation of the Cornales clade. The present study supports the classification within Cornales proposed in Xiang et al. (2002): a Cornaceae of *Cornus*-*Alangium*, a Nyssaceae consisting of *Nyssa*, *Davidia*, and *Camptotheca*, a Mastixiaceae consisting of *Mastixia* and *Diplopanax*, a Grubbiaceae including *Curtisia* and *Grubbia*, Hydrangeaceae, Loasaceae, and Hydrostachyaceae. Given that Alangiaceae has long been widely used and there are also many morphological differences between *Alangium* and *Cornus* (e.g., leaf arrangement nearly always opposite for *Cornus*, alternate for *Alangium*; stamens isomerous with the perianth in *Cornus*, but mostly 2–4 times of perianth parts in *Alangium*; and inflorescence mostly terminal in *Cornus*, but mostly lateral in *Alangium*), it is more desirable to maintain *Cornus* and *Alangium* as two distinct families. Our study also indicated the following: (1) increased sampling of *Hydrostachys* species reduced its long branch length substantially; (2) combining data significantly increased bootstrap support and CI value; (3) major discrepancies between parsimony and maximum likelihood analyses were found regarding the placement of long-branched taxa (e.g., *Hydrostachys*).

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