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Phylogenetic relationships of Combretoidae (Combretaceae) inferred from plastid, nuclear gene and spacer sequences

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Abstract Phylogenetic relationships of the subfamily Combretoidae (Combretaceae) were studied based on DNA sequences of nuclear ribosomal internal transcribed spacer (ITS) regions, the plastid *rbcL* gene and the intergenic spacer between the *psaA* and *ycf3* genes (PY-IGS), including 16 species of eight genera within two traditional tribes of Combretoidae, and two species of the subfamily Strephonematoideae of Combretaceae as outgroups. Phylogenetic trees based on the three data sets (ITS, *rbcL*, and PY-IGS) were generated by using maximum parsimony (MP) and maximum likelihood (ML) analyses. Partition-homogeneity tests indicated that the three data sets and the combined data set are homogeneous. In the combined phylogenetic trees, all ingroup taxa are divided into two main clades, which correspond to the two tribes Lagunculariae and Combretae. In the Lagunculariae clade, two mangrove genera, *Lumnitzera* and *Laguncularia*, are shown to be sister taxa. In the tribe Combretae, two major clades can be classified: one includes three genera *Quisqualis*, *Combretum* and *Calycopteris*, within which the monophyly of the tribe Combretae sensu Engler and Diels including *Quisqualis* and *Combretum* is strongly supported, and this monophyly is then sister to the monotypic genus *Calycopteris*; another major clade includes three genera *Anogeissus*, *Terminalia* and *Conocarpus*. There is no sup-

port for the monophyly of *Terminalia* as it forms a polytomy with *Anogeissus*. This clade is sister to *Conocarpus*.

Key words Combretoidae · Combretaceae · Mangrove · Phylogeny · *psaA*-*ycf3* spacer · *rbcL* gene

The family Combretaceae comprises approximately 600 species in 20 genera of trees, shrubs, and lianas distributed in tropical and subtropical regions, especially in Africa and very often in savannas. *Combretum* Loefl. is the largest genus of this family, containing about 250 species. Many species in the family have economic value. For example, about 200 woody species of *Terminalia* L. are used as resources in the timber, pharmaceutical, and leather industries (Srivastav 1993). The family also includes four mangrove species in three genera *Lumnitzera* Willd., *Laguncularia* Gaertn. f. and *Conocarpus* L.

The family has traditionally been placed in the order Myrtales since Robert Brown established it in 1810 (Dahlgren and Thorne 1984; Cronquist 1988; Tahktajan 1997), and this treatment has largely been supported by recent molecular studies (Conti et al. 1996, 1997; Angiosperm Phylogeny Group 1998). Within the family, two subfamilies, Strephonematoideae and Combretoidae, have been recognized since Engler and Diels (1899). The subfamily Strephonematoideae contains a single genus of six species distributed in western Africa. Their common morphological characteristics include a half inferior ovary and seeds with massive, hemispheric cotyledons. The subfamily Combretoidae contains 19 genera, including the mangrove species, and is characterized by a wholly inferior ovary and seeds with small, folded, and spirally twisted cotyledons.

The subfamily Combretoidae is a taxonomically and phylogenetically complex group. Morphologically, species of Combretoidae are not always easy to recognize because of their wide variation in flowers, fruits, and vegetative shoot morphology (Chao 1958; Stace 1965). Indeed, the classification of Combretoidae has long been controversial (Bentham and Hooker 1867; Brandis 1898; Engler and Diels 1899; Exell 1931; Exell and Stace 1966). For example, Engler and Diels (1899) classified Combretoidae into four

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tribes: Combreteae, Terminaliae, Calycopterideae, and Laguncularieae. Exell (1931) emphasized that the tribe Laguncularieae is a more distinct group than the other three tribes, and Exell and Stace (1966) therefore merged the three tribes into the tribe Combreteae and retained the Laguncularieae as distinct. Recent studies based on palynological data suggested that a revised taxonomic treatment of Combretoideae is still needed (summarized in El Ghazlai et al. 1998). More importantly, because no congruent interpretation on Combretoideae could be found based on the available morphological data, multiple molecular evidence is necessary for inferring phylogenetic relationships of this subfamily.

Sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA) and the *rbcL* gene of chloroplast DNA (cpDNA) have been widely used for phylogenetic studies of angiosperms (e.g., Chase et al. 1993; Baldwin et al. 1995; Hodgkinson et al. 2000). The intergenic spacer between the *psaA* and *ycf3* genes (PY-IGS), which is located from 43410 to 44297 in the cpDNA of tobacco (*Nicotiana tabacum*; Shimada and Sugiura 1991), has also begun to be applied in molecular phylogenetics of Altingiaceae (Shi et al. 2001) and Lythraceae (Huang and Shi 2002). Although conflicts between nrDNA and cpDNA phylogenetics have been reported in some groups (Soltis and Kuzoff 1995; Mason-Gamer and Kellogg 1996), sometimes stronger hypotheses of molecular phylogeny of a particular group can be obtained by combining the sequences of nrDNA and cpDNA for an integrative phylogenetic analysis (Sang et al. 1997; Shi et al. 2002). In this study, phylogenetic analyses using sequences of the nrDNA ITS regions, the cpDNA *rbcL* gene, and the PY-IGS spacer as well as combined data set were carried out to reconstruct a molecular phylogeny of the subfamily Combretoideae.

Materials and methods

Leaves of 19 samples that included 16 species were sampled representing eight of the 19 genera from two tribes of Combretoideae, following the classification of Exell and Stace (1966). The samples were collected from natural or cultivated populations in China, including all six genera from Asia and two mangrove genera from North America (Table 1). Two species of the subfamily Strephonematoideae, *Strephonema pseudocola* and *S. mannii* from Africa, were selected as outgroups.

Total DNAs were isolated from fresh and silica-dried material using the CTAB method (Doyle and Doyle 1987). The nrDNA ITS regions (including ITS1 and ITS2 spacer regions and the 5.8S gene) were amplified following Wen and Zimmer (1996). The primers for amplifying the ITS regions were ITS4 and ITS5 and for sequencing they were C5.8S, ITS4, N5.8S and N18L18 (Wen and Zimmer 1996). Partial fragments of the *rbcL* gene were obtained using the primers P1f (5'-ATG TCA CCA CAA ACA GAG ACT-3') and P2r (5'-CCT TCA TTA CGA GCT TGC ACA C-3') for PCR amplification (Huang and Shi 2002). The same primers

were used for sequencing. For PY-IGS sequences, PCR was performed using PG1f and PG2r, both for amplifying and sequencing under conditions similar to those for ITS amplification. PG1f is located 51–81 bp upstream from the 3' end of the *psaA* gene (5'-cat tcc tcg aac gaa gtt ttg acg gga tcc-3'). PG2r is 28–58 bp downstream from the 5' end of the *ycf3* gene (5'-TCC CGG TAA TTA TAT TGA AGC GCA TAA TTG-3'; Huang and Shi 2002). Amplification products were purified using the QIAquick PCR Purification Kit (CN 28104, QIAGEN), following protocols provided by the manufacturers. Sequencing was done using the Automated Sequencer 377 (Applied Biosystems, Calif). DNA strands of partial samples were sequenced manually using the Sequenase Version 2.0 DNA sequencing kit (US70770, Amersham) and alpha 35S-dATP as a radioactive tracer. All sequences have been submitted to GenBank (for accession numbers, see Table 1).

The DNA sequences were assembled and the boundaries of the *rbcL* gene and PY-IGS region were determined by comparison with the DNA sequences of *Nicotiana tabacum* (Shinozaki et al. 1986; Shimada and Sugiura 1991). The DNA sequences of the ITS regions were compared with that of carrot (*Daucus carota*; Yokota et al. 1989). The assembled sequences and related sequences published in GenBank were aligned using the Clustal-X program (Thompson et al. 1997).

For each data set, maximum parsimony (MP) analysis was performed using a branch-and-bound search using PAUP* 4.0b5 (Swofford 1999). Strict consensus trees were constructed from all most-parsimonious trees. Bootstrap analyses were carried out with 1,000 replicates using TBR branch-swapping of the heuristic search with random taxon addition (100 replicates; Felsenstein 1985). Characters were assigned equal weights at all nucleotide positions (Fitch 1971). Gaps were treated as missing data. Sequence divergences were estimated using Kimura two-parameter distance (Kimura 1980).

Maximum likelihood (ML) analysis was preformed by using the quartet sampling and NJ parameter estimation procedure of Tree-Puzzle 5.0, which implements a fast tree search algorithm, quartet puzzling, and allows analysis of large data sets and automatically assigns estimations of support to each internal branch (Strimmer and von Haeseler 1996, 1997). Three different models of nucleotide substitution, the TN model (Tamura and Nei 1993), the HKY model (Hasegawa et al. 1985), and the SH model (Schoeniger and von Haeseler 1994) were examined. Rate heterogeneity was taken into account by considering invariable sites and by introducing γ -distributed rates for the variable sites. For each data set, a ML search was performed by using parameters estimated from the data with 10,000 puzzling steps.

The MP and ML analyses were performed for data sets (1) ITS, (2) *rbcL*, (3) PY-IGS, and (4) the combined data set. Before combining the data sets, data congruence was assessed with the partition homogeneity test (Farris et al. 1995), implemented with PAUP*4.0b5. One thousand replicates were performed and the resulting *P*-value was used to determine if the use of the combined data set for phylogenetic reconstruction was appropriate.

Table 1. Vouchers, sources, and accession numbers for taxa of plants used. SYS Zhongshan (Sun Yatsen) University, Guangzhou, P.R. China. Classification of the subfamily Combretoideae follows Exell and Stace (1966)

Taxa	Tribe	Subtribe	Species	Source and voucher	Accession no.		
					ITS	rbcL	PY-IGS
Combretoideae	Combretinae	Laguncularieae	<i>Laguncularia racemosa</i> (1)	Cult., Guangdong Inst. Forest., P.R. China, <i>D. Zheng</i> 1011 (SYS)	AF425685	AF425715	AF425701
			<i>Laguncularia racemosa</i> (2)	Cult., Qinglangang, Hainan, P.R. China, <i>X. Ge</i> 1015 (SYS)	AF425686	AF425716	AF425702
			<i>Lumnitzera littorea</i>	P.R. China, Hainan, Dongshaigang, <i>S. Chen</i> 480 (SYS)	AF160468	AF425718	AF425704
			<i>Lumnitzera racemosa</i>	P.R. China, Hainan, Dongshaigang, <i>S. Huang</i> 456 (SYS)	AF160467	AF425717	AF425703
			<i>Combretum alfredii</i>	P.R. China, Lipu, Guangxi, <i>S. Tang</i> 386 (SYS)	AF160471	AF425707	AF425690
		Terminaliinae	<i>Combretum wallichii</i>	Cult., Kunming Bot. Gard., P.R. China, <i>Shi</i> 505 (SYS)	AF208731	AY036151	AY035743
			<i>Quisqualis indica</i> (1)	Cult., Kunming Bot. Gard., P.R. China, <i>Shi</i> 506 (SYS)	AF160470	AF425705	AY035744
			<i>Quisqualis indica</i> (2)	P.R. China, Lingchuan, Guangxi, <i>S. Tang</i> 379 (SYS)	AF425687	AF425705	AF425688
			<i>Quisqualis caudata</i>	Cult., Xishuangbanna Bot. Gard., P.R. China, <i>Y. Huang</i> 360 (SYS)	AF160469	AF425706	AF425689
			<i>Calycopteris floribunda</i>	P.R. China, Yunnan, Yingjiang, <i>X. Gong</i> 759 (SYS)	AF334770	AF281478	AF425691
Strephonematoideae		Strephonematoideae	<i>Anogeissus leiocarpus</i>	P.R. China, Jianfengling, Hainan, <i>S. Huang</i> 443 (SYS)	AF334766	AF425709	AF425693
			<i>Anogeissus acuminata</i>	P.R. China, Yingjiang, Yunnan, <i>X. Gong</i> 628 (SYS)	AF334765	AF425708	AF425692
			<i>Conocarpus erectus</i>	Cult., Qinglangang, Hainan, P.R. China, <i>X. Ge</i> 1014 (SYS)	AY050562	AF281477	AF425700
			<i>Terminalia arjuna</i>	Cult., South China Bot. Gard., P.R. China, <i>S. Jian</i> 387 (SYS)	AF338255	AF425711	AF425695
			<i>Terminalia muelleri</i> (1)	P.R. China, Jianfengling, Hainan, <i>W. Liao</i> 398 (SYS)	AF160472	AF425712	AF425697
			<i>Terminalia muelleri</i> (2)	Cult., Kunming Bot. Gard., P.R. China, <i>Y. Huang</i> 363 (SYS)	AF334767	AF425713	AF425698
			<i>Terminalia bellirica</i>	Cult., Kunming Bot. Gard., P.R. China, <i>Y. Huang</i> 364 (SYS)	AF334768	AF425714	AF425699
			<i>Terminalia chebula</i>	P.R. China, Jianfengling, Hainan, <i>S. Huang</i> 442 (SYS)	AF334769	AF425710	AF425696
			<i>Terminalia hainanensis</i>	Cult., Campus of Zhongshan Uni., P.R. China, <i>C. Ye</i> 381 (SYS)	AF160466	AY050563	AF425694
			<i>Strephonema pseudocola</i> ^a	Cult., Korup National Park, Ndian River Delta, Cameroon, <i>M. Sainge & P. Mambo</i> 823	AF508244	AF508248	AF508246
			<i>Strephonema manni</i> ^a	Cameroon, Mundemba, Ndian River Delta, Mundemba, Cameroon, <i>M. Sainge & P. Mambo</i> 807	AF508249	AF508247	AF508245

^aOutgroups

Results

The total length of the ITS1, 5.8S, and ITS2 regions of the 19 ingroup samples (three of them represent the same species) ranged from 601 bp to 615 bp with an ITS1 of 215–245 bp, a 5.8S of 163–167 bp and an ITS2 of 203–222 bp. The aligned sequences were 657 bp long with 315 variable characters, of which 269 characters were parsimony-informative (Table 2). Sequence divergence in the ITS regions between the ingroup genera ranged from 9.6% (between *Quisqualis*

L. and *Combretum*) to 25.0% (between *Combretum* and *Laguncularia*).

The rbcL sequences from the ingroup species comprised 1,258 nucleotide sites. Of the 1,258 sites, 86 were variable and 58 were parsimony-informative (Table 2). The proportion of nucleotide differences ranged from 0.0% to 2.2% within the ingroup samples.

The sequences of the PY-IGS region obtained from the 19 ingroup samples ranged from 693 to 708 bp in length. The aligned sequences contained 757 sites, of which 131 were variable and 118 were parsimony-informative (Table 2).

Table 2. Comparison of indices for the most parsimonious trees (MPTs) of Combretoidae based on ITS, *rbcL* and PY-IGS sequences, as well as the combined data set. Tree lengths include uninformative characters; CI consistency index, excluding uninformative characters; RI retention index; RC re-scaled consistency index

Data set	No. variable characters	No. informative characters	No. of trees	Length of trees	CI	RI	RC
ITS	315	269	1	725	0.6290	0.7413	0.4663
<i>rbcL</i>	86	58	26	113	0.8230	0.8621	0.7095
PY-IGS	131	118	10	145	0.9379	0.9548	0.8955
ITS + <i>rbcL</i> + PY-IGS	532	445	1	968	0.7076	0.7955	0.5629

Sequence divergence ranged from 0.0% to 3.6% among ingroup taxa.

The *P* values resulting from the partition-homogeneity tests are shown in Table 3. The cpDNA *rbcL* and PY-IGS sequences were congruent (*P* = 1.000). The cpDNA *rbcL* + PY-IGS and nrDNA ITS sequences were also congruent at the 5% level (*P* = 0.053). The combined data set, containing the 19 ingroup samples from ITS, *rbcL*, and PY-IGS sequences, resulted in a matrix of 2,672 nucleotide characters with 532 variable sites and 445 parsimony-informative sites (Table 2).

The numbers of equally most parsimonious trees (MPTs) based on the four data sets were 1 (ITS), 26 (*rbcL*), 10 (PY-IGS), and 1 (the combined data). The lengths, consistency indices (CI), retention indices (RI), and re-scaled consistency indices (RC) of the trees are also shown in Table 2. The MP analysis based on the combined data set resulted in a single fully resolved tree with higher bootstrap support values for most branches than those produced by the analysis of each separate data set (data not shown; Fig. 1). The ML analysis based on separate and combined data and different nucleotide substitution models and rate heterogeneity models resulted in a quartet puzzling (QP) tree that had the same topology to those from the MP (Fig. 1). Seven of the eight genera are shown to be monophyletic whereas the monophyly of *Terminalia* cannot be determined from these data because *Anogeissus* Wall. ex Gurlen. et Perr. was found within an unresolved *Terminalia* clade. Both the combined MP tree and the QP tree support two major clades: one comprises the Lagunculariae species with 91% bootstrap support for the MP tree and 96% for the QP tree; the other clade, which is correspondent to the tribe Combretaeae sensu Exell and Stace, consists of the six remaining genera (MP support value = 73% and QP support value = 95%).

Discussion

Individual versus combined data sets

Although the three molecular markers originate from different genomes and regions (chloroplast or nuclear genome, coding, or spacer region), their congruence is revealed by the partition homogeneity tests (Table 3). Testing congruence between individual data sets is one of the most fundamental approaches to determining the reliability of phylogenetic inferences (de Queiroz et al. 1995; Miyamoto

Table 3. *P* values from partition-homogeneity test with 1,000 replications for various partitions of the data

Data sets	<i>P</i> value
ITS vs <i>rbcL</i>	0.582
ITS vs PY-IGS	0.302
<i>rbcL</i> vs PY-IGS	1.000
<i>rbcL</i> /PY-IGS vs ITS	0.053

and Fitch 1995; Huelsenbeck et al. 1996). As in other work involving the combination of multiple molecular data sets (Soltis et al. 1998; Hoot et al. 1999), our study indicates that the combined analysis resulted in a higher degree of resolution than that when the data sets were analyzed alone. Therefore, the following discussion is mainly based on the results obtained from the combined analysis.

Outgroups

The African genus *Strephonema*, originally placed in Lythraceae (Bentham and Hooker 1867) and several other families (Outer and Fundter 1976), was first included in Combretaceae as the only genus of subfamily Strephonematoideae (Engler and Diels 1899). Stace (1965) suggested that the possession of typical combretaceous compartmented hairs in *Strephonema* species indicated the affinity of this genus with the Combretaceae. However, their semi-inferior ovary and unique features in their paracytic subsidiary cells and revolute domatia supported the treatment of *Strephonema* as a separate subfamily of the Combretaceae. In this study, therefore, we chose *Strephonema pseudocola* and *S. manni* as outgroups for reconstructing the phylogeny of Combretoidae.

Phylogenetic relationship within Combretoidae

Both the combined MP tree and QP tree demonstrate that the ingroup taxa should be divided into two main clades within Combretoidae: one includes two genera, *Lumnitzera* and *Laguncularia*, which correspond to the tribe Lagunculariae, well supported by both parsimony and maximum likelihood (Fig. 1); another main clade consists of the remaining taxa, which belong to Combretaeae (73% bootstrap value support in the MP tree and 99% in the QP tree). This relationship was suggested by Exell and Stace (1966), who combined the three tribes, Combretaeae,

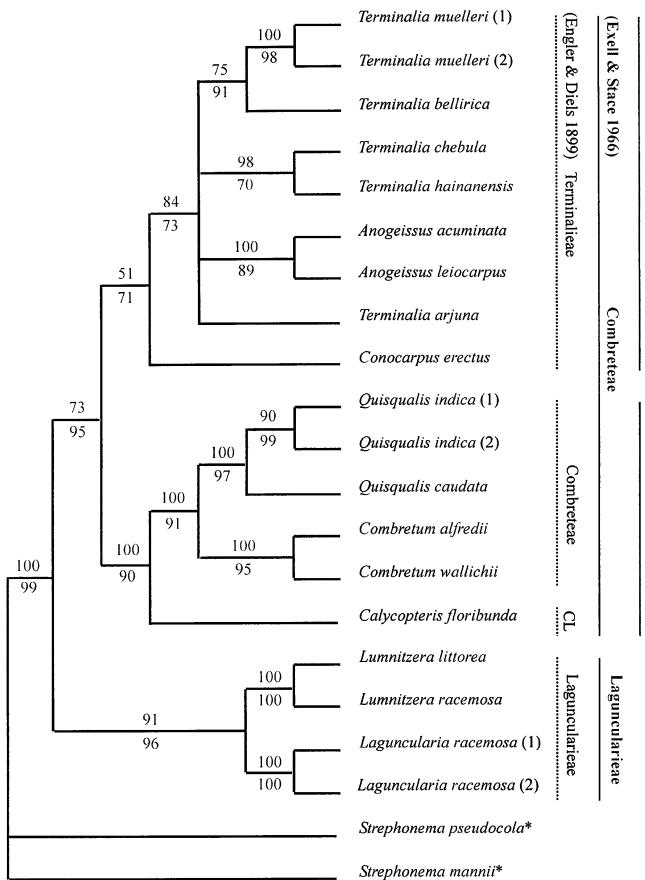


Fig. 1. Strict consensus tree of equally most parsimonious trees of the subfamily Combretoidae generated using maximum parsimony (MP) and maximum likelihood (ML) analyses based on combined molecular data. Numbers above branches represent bootstrap values (%) for the clades with 1,000 replicates based on the MP analysis, and below for the QP analysis. Asterisks indicate outgroups used to root trees. CL Calycopterideae

Terminaliinae, and Calycopterideae originally proposed by Engler and Diels (1899) into one tribe, Combretaceae, and retained the Lagunculariaeae based on morphological and anatomical characters. The possession of a pair of bracteoles adnate to the lower receptacle and the lack of a visible venation pattern on the upper epidermis is the most distinctive feature of Lagunculariaeae. In contrast, species of Combretaceae sensu Exell and Stace (1966) often have a bracteole-adnate receptacle, with a venous system on the leaves.

Mangrove genera of the family are predominately placed in the tribe Lagunculariaeae. *Lumnitzera* includes two species, *L. littorea* and *L. racemosa*, both of which play important roles in the mangrove forests of tropical Asia and Africa. *Laguncularia racemosa*, the white mangrove, is the only species of *Laguncularia*, often distributed in littoral regions of tropical and subtropical America and Western Africa. Contrasting to some other mangroves, it does not develop prop roots or pneumatophores. In this study, each of the two genera forms a strongly supported monophyletic

group based on the combined data (100% bootstrap under both optimization approaches, Fig. 1).

Within the tribe Combretaceae, three subtribes, Pteleopsidinae, Combretinae and Terminaliinae, were proposed by Exell and Stace (1966). The latter two were examined in this study. The results from our molecular data strongly support the monophyly of subtribe Combretinae with high bootstrap support (Fig. 1). Within the clade, *Quisqualis* and *Combretum* are sisters and both are then sister to *Calycopteris* Lam., with high bootstrap support values (Fig. 1). Engler and Diels (1899) emphasized the morphological differences among *Quisqualis*, *Combretum*, and *Calycopteris*, and then put them into two tribes: Combretaceae (*Quisqualis* and *Combretum*) and Calycopterideae (*Calycopteris*). The latter consists of a monotypic genus *Calycopteris*, and its macroscopic or epidermal character differs from the other genera of Combretaceae (Stace 1965; Exell and Stace 1966). Although the sister group relationship between *Calycopteris* and the other members of Combretaceae sampled in this study was strongly supported, their ranking as two separate subtribes is not comparable with ranking in their sister clade Terminaliinae (Fig. 1).

The concept of *Quisqualis* has had a varied history (Bentham and Hooker 1867; Brandis 1898; Engler and Diels 1899), later stabilized by Exell (1931), who separated it from the large genus *Combretum* based on its fused-to-the-hypanthium stamens. Although sampling in the two genera in this study is insufficient to examine problems of the circumscriptions of *Combretum* and *Quisqualis*, our results show that the sampled species from the two genera form monophyletic groups, in accordance with their morphological evidence. In addition, of all the studied genera of Combretoidae, the lowest sequence divergence (2.7%) of the combined sequence data was found between these two genera. Therefore, both morphological and molecular data confirm a close relationship between *Combretum* and *Quisqualis*.

The remaining genera *Terminalia*, *Anogeissus*, and *Conocarpus* belong to the subtribe Terminaliinae (Exell and Stace 1966). The number of genera recognized within Terminaliinae based on morphology has varied with different authors, even though certain genera are well defined. In this study, the two species of *Anogeissus* form a monophyletic group, but there is no support for a monophyletic *Terminalia* since it forms a polytomy with *Anogeissus* (Fig. 1). Species of *Terminalia*, the second largest genus of the Combretaceae, vary greatly in morphology, anatomy, and karyotype evidence (Exell 1954; Stace 1965; Exell and Stace 1966; Ohri 1996). Many taxonomic problems remain within this genus, which has never been satisfactorily classified into subgenera and sections.

A clade comprising the mangrove genus *Conocarpus* and genera of Terminaliinae is found in both analyses, but with low bootstrap support (Fig. 1). *Conocarpus erectus*, the so-called button mangrove, is not a true mangrove, and is often found as an invader in tropical America and tropical West Africa mangrove forests. Compared with the other two mangrove genera of the subfamily, *Conocarpus* shares a number of epidermic features with *Lumnitzera* and *Laguncularia*.

cularia. However, its stalked glands, the typical domatia and the presence of a lateral venous system on the epidermises show good evidence for placing them in separate tribes (Stace 1965). Furthermore, the conspicuous lebetiform domatia in *Conocarpus* indicate that it belongs to the Terminaliinae rather than the Combretinae. Our results thus agree with the traditional morphological classification.

Subtribe Pteleopsidinae contains a single genus, *Pteleopsis*. Samples of this African genus, though important, were excluded in this study. Indeed, the central distribution of Combretaceae is confined to Africa, which led to the difficulty of getting fresh samples of some genera of this family. Although some have been obtained from herbarium specimens, PCR amplification was unsuccessful because most of the total DNA extracted from specimens was too degraded. More genera and species of Combretoideae should be included in further studies, perhaps using modified techniques (e.g., Huang et al. 2002) or by collecting fresh material to analyze their phylogenetic relationships.

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