Molecular Phylogenetics of Subfamily Calamoideae (Palmae) Based on nrDNA ITS and cpDNA *rps*16 Intron Sequence Data

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Phylogenetic relationships among the 22 genera of the palm subfamily Calamoideae were investigated using DNA sequence data from the nuclear ribosomal internal transcribed spacer (ITS) region and the chloroplast *rps*16 intron. The *rps*16 intron displayed low levels of variation, corroborating previous reports that the chloroplast genome of palms is highly conserved. High levels of within-individual polymorphism were identified in the ITS region, indicating that concerted evolution is not effectively homogenizing the ITS repeats. In the majority of cases, multiple clones from individuals resolved as monophyletic. However, the high levels of homoplasy in the ITS dataset, along with generally poor jackknife support for many clades, led to concerns that topologies obtained from these data might be unreliable. Nevertheless, congruence between trees based on ITS data alone and those based on *rps*16 intron data was high. Simultaneous analyses of both datasets yielded well-resolved topologies with high levels of jackknife support. A number of exciting groups emerged from the analyses: the African rattan clade comprising the endemic African rattan genera *Laccosperma, Eremospatha,* and *Oncocalamus;* the Lepidocaryeae–*Raphia* clade comprising the fan-leaved New World tribe Lepidocaryeae and the African genus *Raphia;* and the Asian clade comprising all Asian genera except *Eugeissona.* The position of *Eugeissona* was variable, although it did not resolve inside any of the three major clades mentioned above. © 2000 Academic Press

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INTRODUCTION

The Calamoideae is a large, pantropical subfamily within the palm family (Palmae or Arecaceae) compris-ing 22 genera and approximately 650 species, divided among two tribes and eight subtribes (Uhl and Drans-field, 1987). Although the subfamily contains tree palms and acaulescent palms, it is perhaps best known for its climbing members, the rattans, which are highly di-verse in the forests of South-East Asia in particular and are of economic importance as a source of raw material for the cane-furniture industry.

Recent phylogenetic analyses of morphological and molecular data for the entire palm family strongly support the monophyly of the Calamoideae (Asmussen *et al.,* 2000; Baker *et al.,* 1999a; Uhl *et al.,* 1995). However, relationships within the Calamoideae are difficult to interpret in view of the wide spectrum of morphological diversity that is encompassed by the subfamily. Analyses of morphological data for subfam-ily Calamoideae alone failed to yield a well-supported phylogeny (Baker *et al.,* 1999b). Thus, for alternative, independent estimates of phylogeny to be obtained, a further source of data, such as DNA sequence, is required.

Molecular systematic research of the Palmae is af-fected by one general quality of palm molecular evolu-tion: palm DNA evolves slowly. Substitution rate esti-mates from restriction site variation in chloroplast DNA were found to be 5- to 13-fold slower than rate estimates for grasses (Wilson *et al.,* 1990). A similar study of substitution rates in the chloroplast-encoded gene *rbc*L confirmed this finding by identifying a substi-tution rate in palms that is five times slower than that of grasses (Gaut *et al.,* 1992). A further investigation, this time focusing on the multicopy nuclear gene *Adh,* revealed a synonymous substitution rate in palms that is approximately 2.5-fold slower than that of grasses (Gaut *et al.,* 1996). Comparisons of substitution rates at nonsynonymous sites were more complex. The nonsyn-onymous rate for the palm *Adh*A locus was intermedi-ate between the rates calculated for the two paralogous loci identified in grasses, *Adh*1 and *Adh*2. Unfortu-nately, data for the two other *Adh* loci known in palms, *Adh*B and *Adh*C, were not available. Additional data from the mitochondrial gene *atp*A yielded a conclusion

similar to that obtained from the *Adh* data; synonymous substitution rates in palm mitochondrial DNA are lower than those in grasses (Eyre-Walker and Gaut, 1997). Moreover, the relative rates of synonymous substitution between palms and grasses were shown to be similar at the three loci *Adh, rbc*L and *atp*A, indicating a correlation in synonymous site evolution across genomes (Eyre-Walker and Gaut, 1997). Given this correlation, the underlying cause of slow evolution in palm DNA might be attributed to a single evolutionary process that affects all three genomes. For example, mutation rates are affected by generation times and it has been noted that substitution rates in *rbc*L in monocotyledons are inversely correlated with minimum generation time (Eyre-Walker and Gaut, 1997; Gaut *et al.,* 1992, 1996).

The peculiarities of palm molecular evolution discussed above limit the choice of well-known DNA regions that can be used for answering systematic questions within the family. For example, the *trn*L intron and the *trn*L–*trn*F spacer of the chloroplast genome have been successfully used to investigate relationships within genera in some angiosperm families (e.g., *Gentiana* (Gentianaceae), *trn*L intron only (Gielly and Taberlet, 1996), *Actaea* (Ranunculaceae) (Compton *et al.,* 1998)). In contrast, a current phylogenetic study of the entire palm family has revealed very low levels of informative base substitution in these regions. While analysis of the data yields some resolution among palm genera, the region is almost completely uninformative for phylogeny reconstruction within subfamily Calamoideae (Baker *et al.,* 1999a). Barrow (1998) conducted a molecular systematic study of the genus *Phoenix* (Coryphoideae, Phoeniceae) using sequence data from the nontranscribed spacer of the nuclear 5S rDNA repeat regions. However, a short pilot study of the Calamoideae indicated that the 5S nontranscribed spacer is too divergent to be alignable across all taxa (W. J. Baker, unpublished), although it has proved useful for lower taxonomic levels in the subfamily (Baker *et al.,* 2000). The low rate of nucleotide substitution observed in palms imposes limits on the value of many well-characterized DNA regions that have been used at lower taxonomic levels in other angiosperm groups. Restriction fragment length polymorphism (RFLP) data have been used successfully for phylogenetic reconstruction in the Palmae (Hahn, 1993; Uhl *et al.,* 1995). However, a sequencing approach has been pursued here, despite the difficulties discussed, because a suitable DNA sequence can provide more characters which can be collected with greater ease and less expense and which exhibit fewer problems of homology assessment than RFLPs.

The study detailed in this paper exploits two DNA regions that have not been used previously for phylogeny reconstruction in the Palmae. The first is the internal transcribed spacer (ITS) region of the 18S–26S nuclear ribosomal cistron (18S–26S nrDNA). The 18S–26S nrDNA comprises three genes which code for the 18S, 5.8S, and 26S ribosomal subunits. The three genes are separated by two internal transcribed spacers: ITS1 between 18S and 5.8S, and ITS2 between 5.8S and 26S. Each spacer is typically less than 300 bp long in angiosperms (Baldwin *et al.,* 1995). Many thousands of copies of the 18S–26S nrDNA may be present in tandem repeats in the plant nuclear genome, each cistron being separated by an intergenic spacer (Baldwin *et al.,* 1995). The existence of multiple loci for the tandem repeats is well documented (e.g., Badaeva *et al.,* 1996; Thomas *et al.,* 1997). For example, in diploid *Aegilops* (Gramineae) species, one or two loci were found in nucleolus organiser regions, with up to nine additional minor loci identified on different chromosomes (Badaeva *et al.,* 1996).

The use of the ITS region in plant molecular systematics has been reviewed by Baldwin *et al.* (1995). The ITS region is now a widely used data source in molecular systematic studies of plants at lower taxonomic levels for two principal reasons. First, the high copy number allows easy amplification of the region from total DNA. Second, the spacer sequences evolve rapidly and can therefore resolve lower level relationships better than slowly evolving genes, such as 18S and *rbc*L (Baldwin, 1992; Baldwin *et al.,* 1995). The rapid evolution in the ITS region is often attributed to a lack of functional constraints. However, there is strong evidence that the two spacers have a role in the maturation of the ribosomal subunits (Baldwin *et al.,* 1995 and references therein). Some portions of ITS1 and ITS2 are conserved across all angiosperms, and phenograms derived from alignments of ITS2 regions from a wide range of angiosperm taxa reflect some accepted higher-level relationships (Hershkovitz and Zimmer, 1996; Hershkovitz and Lewis, 1996). These conserved regions may be involved in the formation of secondary structure during the processing of the cistron transcript (Baldwin *et al.,* 1995).

Given the large number of tandem repeats of 18S–26S nrDNA in the nuclear genome, it is remarkable that, in many cases, there is considerable homogeneity among different copies within individuals. The homogenization of repeating DNA is effected by concerted evolution, a phenomenon comprising processes such as gene conversion and unequal crossing over, which can alter gene frequencies in daughter chromosomes (Arnheim, 1983; Hillis *et al.,* 1991; Wendel *et al.,* 1995). In cases in which homogenization is complete (e.g., Compton *et al.,* 1998), it is possible to reconstruct phylogenies without difficulty. However, in cases in which homogenization is incomplete (e.g., Buckler and Holtsford, 1996a; Buckler *et al.,* 1997), phylogeny reconstruction may not be straightforward. Where intragenome polymorphism is identified in multigene families, it is necessary to isolate multiple copies from individuals by cloning and

to analyze all sequences simultaneously. The interpretation of gene trees from such analyses can be problematic when the impact of concerted evolution is intermediate, as the high levels of homoplasy that occur in such circumstances may result in unreliable phylogeny reconstruction (Sanderson and Doyle, 1992).

The second DNA region used for investigating calamoid phylogeny in this study is a group II intron located between the two exons of *rps*16, a gene found in the large single-copy region of the chloroplast genome that codes for ribosomal protein small subunit 16. The region (intron and exons) is reported to be completely or partially missing in some members of a variety of angiosperm families, including Linaceae, Malpighiaceae, Passifloraceae, Salicaceae, Polygalaceae, Turneraceae, Violaceae, Connaraceae, Eucommiaceae, Fagaceae, and Leguminosae (Downie and Palmer, 1992; Doyle *et al.,* 1995). The *rps*16 intron is somewhat length variable, ranging from 707 to 951 bp in the Caryophyllaceae alone (Oxelman *et al.,* 1997). The few published phylogenetic studies that use the *rps*16 intron indicate that it displays levels of sequence divergence between two and three times lower than those of the ITS region and that it may therefore be a valuable source of data for taxonomic studies above species but below the family level (Lidén *et al.,* 1997; Oxelman *et al.,* 1997).

MATERIALS AND METHODS

Ingroup and Outgroup Sampling

The sampling strategy employed in this study resembled closely that used in the phylogenetic analyses of calamoid morphology of Baker *et al.* (1999b). All calamoid tribes, subtribes, and genera are represented in the sample (Appendix 1), and where noteworthy morphological variation exists within genera, multiple representatives of those genera were included. Outgroups included in the study were chosen from four of the five other palm subfamilies: *Kerriodoxa elegans* (Coryphoideae), *Ceroxylon quindiuense* (Ceroxyloideae), *Asterogyne martiana* (Arecoideae), and *Nypa fruticans* (Nypoideae). *N. fruticans* was incorporated in the *rps*16 dataset but not in the ITS dataset, as sequences obtained could not be aligned. In total, 40 species were included in the ITS dataset and 41 species in the *rps*16 intron dataset.

DNA Extraction, Amplification, Cloning, and Sequencing

Total DNA was extracted from fresh or silica gel-dried leaf material (Chase and Hills, 1991) using either a large-scale CTAB protocol (Doyle and Doyle, 1988) or the Qiagen DNeasy Plant Mini Kit. The latter method was found to give a much cleaner product than that of Doyle and Doyle (1988), although usually lower concentrations of DNA were obtained. Locations of voucher specimens for each DNA extraction are indicated in Appendix 1.

The ITS region and the *rps*16 intron were amplified from total DNA using the polymerase chain reaction. The ITS region was amplified using primers 17SE (Sun *et al.,* 1994) and ITS4 (White *et al.,* 1990) (17SE = ACGAATTCATGGTCCGGTGAAGTGTTCG; ITS4 = TCCTCCGCTTATTGATATGC). Primers used for amplification of *rps*16 were rpsF and rpsR2 (Oxelman *et al.,* 1997) (rpsF = GTGGTAGAAAGCAACGTGCGACTT; rpsR2 = TCGGGATCGAACATCAATTGCAAC). One hundred-microliter reactions were prepared (buffer as provided by Promega; 1.5 mM $MgCl_2$, 0.3 μM each primer, 0.1 mM each dNTP, 2.5 units of *Taq* DNA polymerase (Promega), and 1 μl of template DNA) and overlaid with two drops of mineral oil. In general, template DNA was taken directly from the undiluted product of extraction for ITS amplifications, whereas for *rps*16 intron amplifications, a 10× dilution of the extraction product was used as template DNA. The reactions were placed in a Perkin–Elmer thermocycler and exposed to the following PCR profile: denaturing step of 97°C for 1 min, 1 cycle; denaturing step of 97°C for 1 min, annealing step of X°C for 1 min (X = 52 for ITS, 53 for *rps*16 intron), extension step of 72°C for 2 min, 27–30 cycles; final extension step of 72°C for 7 min, 1 cycle; 4°C soak. Reactions were cleaned using the QIAquick PCR purification kit from Qiagen and the purified products eluted into 50 μl of water or 30 μl in the case of weak reactions.

Preliminary investigations suggested that the ITS region of calamoid palms was polymorphic. Direct sequencing of ITS PCR products proved impossible. Therefore, all ITS PCR products were cloned using the pGEM-T Vector System from Promega. Ligations and transformations were prepared according to the protocol provided by the manufacturer. Transformed *Escherichia coli* were spread onto 20-ml agar plates (LB medium, including 100 μg/ml ampicillin, 0.5 mM isopropylthio-β-D-galactoside (IPTG), and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)) and incubated at 37°C overnight. Between 5 and 10 white colonies were selected at random. From each, a scrape of cells was removed with a sterile cocktail stick and suspended in 10 μl of water. Subsequently, 1 μl of cell suspension was substituted for total DNA in a PCR that was otherwise identical to that described above. PCRs were run on an agarose gel and cleaned as detailed above. At least two clones were sequenced for almost every species included in the study. Each clone is represented in the trees described in this paper by the taxon name followed by a number (e.g., *Raphia farinifera*. 4 = clone 4 from *Raphia farinifera*).

Clean PCR products were cycle-sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin–Elmer. Amplification prim-

ers were used as sequencing primers. The *rps*16 intron of *Korthalsia cheb* could not be sequenced with amplification primers only. To solve this problem, three reverse internal primers, rpsR3, rpsR4, and rpsR5, were designed and used as additional sequencing primers (rpsR3 = TCCTCATACGGCTCGAGAA; rpsR4 = TATT-GAGCCGTCTCTAAC; rpsR5 = ATGAACGGTTGATT-CCC).

Raw data files were assembled and edited using SeqMan, part of the DNASTAR Lasergene software package (1994). All sequences of the ITS region were submitted for BLAST searching in GenBank to ensure that no contaminant sequences had been isolated (e.g., endophytic fungi).

Edited sequences were entered into the alignment package MegAlign (Lasergene, DNASTAR). After a small number of sequences had been added, preliminary alignments were generated using the Clustal algorithm as implemented in MegAlign. The alignment was adjusted by eye before all remaining sequences were added. Raw data were checked to verify that variable positions were not merely base-calling errors. Small portions of the outgroup ITS sequences could not be aligned confidently with the ingroup. These ambiguous positions in the outgroup sequences were recoded with Ns (IUPAC ambiguity code for A, C, G, or T) so as to avoid the complete exclusion of the region from analysis. Alignment of some portions of the ITS and *rps*16 datasets was ambiguous. These regions were excluded from all analyses described below. Jukes–Cantor distances were calculated by pairwise comparison between all sequences after exclusion of ambiguously aligned regions. Copies of the alignments in Nexus format, including details of the exclusion sets, are available from the corresponding author.

Cladistic Analyses

Cladistic analyses were conducted using PAUP* versions 4.059 and 4.064 (written by D. L. Swofford) and Parsimony Jackknifer version 4.22 (Farris, 1995; Farris *et al.,* 1996). In all maximum parsimony analyses, parsimony-uninformative characters were excluded and all included characters were unordered. Both datasets were analyzed extensively as detailed below.

ITS analysis 1. Given the large number of terminal taxa in the ITS dataset (108 sequences for 40 species), a complex search strategy was required to improve the probability of recovering optimal trees. The purpose of analysis 1 was to use starting trees, gathered rapidly from different parts of the tree space, in a search employing a rigorous branch-swapping algorithm. In this way, optima can be discovered efficiently without conducting multiple heuristic searches and swapping to completion in each one. Five hundred consecutive heuristic searches were conducted using stepwise addition with random taxon addition for each search, with nearest-neighbor interchange (NNI) branch swapping,

MULPARS, and steepest descent in effect. Branches were collapsed if their maximum length equaled zero. A maximum of four trees was saved during each search, even if these were longer than the overall shortest tree length. These trees were used as starting trees in a subsequent search with tree-bisection–reconnection (TBR) branch swapping, MULPARS, and steepest descent in effect. Again, branches were collapsed if their maximum length equaled zero. To assess clade support, the dataset was analyzed using Parsimony Jackknifer, searching for 10,000 replicates and retaining groups that appeared in 50% or more of the trees. The g_1 statistic (Hillis and Huelsenbeck, 1992) was calculated with PAUP* by evaluating 100,000 random trees. The standardized consistency index (excluding autapomorphies), CI_9', was calculated and used to evaluate S, the maximum probability of correct phylogenetic inference (Givnish and Sytsma, 1997).

ITS analysis 2. To reduce computational difficulties caused by the large number of terminal taxa, the dataset was reduced by excluding sequences so that only 60 sequences were included for the same 40 species. Sequences were selected for exclusion from the large dataset by examining the trees and clade support from analysis 1. Where multiple clones derived from single individuals were resolved as monophyletic groups with jackknife support exceeding 50%, only one sequence was retained as a representative in analysis 2. Two clones of *R. farinifera* were retained, although all eight clones included in ITS analysis 1 were supported by the jackknife, because the intragenome divergence between clones was so high (see Table 3). While jackknife values as low as 50% are very weak evidence of monophyly, the fact that members of the clades concerned were derived from single individuals was considered sufficient supporting evidence in favor of reducing the representation. The reduced dataset was analyzed in 400 consecutive heuristic searches, with starting trees obtained by random taxon addition, TBR swapping, MULPARS, and steepest descent in operation. Jackknife support, g_1, CI_9', and S were assessed as in analysis 1.

ITS analysis 3. The reduced dataset was analyzed using a successive approximations weighting approach (Farris, 1969, 1989; Goloboff, 1993). Using the optimal trees from analysis 2, characters were reweighted by their rescaled consistency indices (reweight by maximum value if more than one tree in memory, base weight = 1000) and subjected to 100 consecutive heuristic searches under the conditions employed in analysis 2. This process was iterated until the topology stabilized. The weight set of the final round of successive approximations weighting was used in a jackknife analysis in PAUP*, searching for 10,000 replicates and retaining groups that appeared in 50% or more of the trees. Options were set so as to emulate the conditions

enforced by Parsimony Jackknifer (Farris, 1995), which does not allow user-specified weighting schemes (collapse branches if minimum length is zero, jackknife with 36.79% deletion, emulate "Jac" resampling, "Fast" stepwise-addition).

ITS analysis 4. A maximum likelihood analysis of the reduced ITS dataset was implemented. Ten trees were selected at random from the equally most-parsimonious trees saved in ITS analysis 2. Likelihood parameters were estimated from these trees (substitution rate matrix with six substitution types under a general time-reversible model, proportion of invariable sites, shape parameter of gamma distribution with four rate categories). The parameters from the most likely of the 10 trees were then fixed in a heuristic search with starting trees obtained by stepwise addition (random addition sequence) and with TBR swapping and MULPARS in effect.

rps16 analysis 1. As the *rps*16 dataset contained fewer terminal taxa than the ITS dataset, searching was more straightforward. All taxa were included in the dataset, which was analyzed using PAUP* and Parsimony Jackknifer as detailed above for ITS analysis 2. The g_1 statistic, CI'_9, and S were calculated as in ITS analysis 1.

rps16 analysis 2. The dataset was analyzed under a regime of successive approximations weighting and jackknife support was calculated as described for ITS analysis 3.

rps16 analysis 3. A maximum likelihood analysis of the *rps*16 dataset was conducted as described in ITS analysis 4. Likelihood parameters were estimated from all the trees found in *rps*16 analysis 1. As some groups of taxa possessed identical sequences for included regions, only one representative was included in the analysis so as to increase computational efficiency. Thus, *Retispatha dumetosa* represented *Calamus thysanolepis* and *C. nanodendron, Metroxylon sagu* represented *M. salomonense,* and *Pigafetta filaris* represented *P. elata.*

Simultaneous analysis 1. A dataset comprising both ITS and *rps*16 intron sequences was constructed. All 38 taxa for which data from both regions were available were included. The ITS clones included in the reduced

ITS dataset were used in the combined dataset. For those taxa represented by multiple ITS clones in the reduced ITS dataset, one clone was selected randomly for inclusion in the combined dataset. The ITS clones incorporated in the combined dataset are indicated in Appendix 1. The dataset was analyzed using the same methods as those implemented in ITS analysis 2. Jackknife support, the g_1 statistic, CI_9', and S were calculated as in ITS analysis 1.

Simultaneous analysis 2. The combined dataset was analyzed under a regime of successive approximations weighting, and jackknife support was calculated as described for ITS analysis 3.

RESULTS

The ITS Region in Calamoid Palms

Amplification of ITS regions using the primer pair described above gave PCR products of approximately 900 bp in length. On comparison with an almost complete sequence for the 18S–26S nrDNA cistron of *Arabidopsis thaliana* (GenBank Accession No. X52320), it was observed that c. 100 bp of each PCR product was derived from the 18S gene. The mean length of the entire ITS region was 671 bp. The mean lengths of ITS1, ITS2, and the 5.8S gene were 266, 243, and 163 bp, respectively (Table 1). The GC content of individual sequences (all positions included) ranged from 53 to 70% (mean = 62%).

Pairwise Jukes–Cantor distances for the ITS dataset are summarized in Table 2. The mean distance for the entire dataset is 0.257 (SD = 0.062, range = 0.000–0.404). Comparisons of multiple clones from individuals revealed high levels of intragenome polymorphism in the ITS regions. The mean intragenome pairwise distance across the entire dataset is 0.102 (SD = 0.095, range = 0.000–0.258). Up to 10 clones were sequenced for several taxa to investigate this phenomenon further; the results are summarized in Table 3. Mean distances ranged from 0.016 (SD = 0.006, range = 0.003–0.026) for 10 clones of *Calamus hollrungii* to 0.212 (SD = 0.053, range = 0.002–0.256) for *R. farinifera.*

TABLE 2

Summary of Pairwise Jukes–Cantor Distances between ITS Sequences

ITS dataset partition	Mean	SD	Range
Total dataset pairwise distances	0.257	0.062	0.000–0.404
Total intergenome pairwise distances	0.261	0.056	0.015–0.404
Total intragenome pairwise distances	0.102	0.095	0.000–0.258

TABLE 1

Summary of Sizes (bp) of ITS Cistron, Individual Spacers, 5.8S Gene, and *rps*16 Intron

Region	Mean	SD	Range
ITS1–5.8S–ITS2	671	37	531–863
ITS1	266	27	171–336
ITS2	243	27	94–433
5.8S	163	4	126–180
*rps*16 Intron	870	26	731–905

TABLE 3

Intragenome Pairwise Jukes–Cantor Distances between ITS Sequences from Five Taxa for Which Five or More Clones Were Sequenced

Taxon	Number of clones compared	Mean	SD	Range
Calamus hollrungii	10	0.016	0.006	0.003–0.026
Pogonotium ursinum	5	0.058	0.064	0.005–0.134
Metroxylon salomonense	5	0.158	0.016	0.128–0.176
Raphia farinifera	8	0.212	0.053	0.002–0.256
Mauritia flexuosa	5	0.160	0.112	0.000–0.251

The rps16 Intron in Calamoid Palms

Amplification products of the *rps*16 intron were approximately 900 bp in length. A sequence for the *rps*16 exons and intron of *Zea mays* (Kanakari *et al.,* 1992; GenBank Accession No. X60823) could be aligned with ease against sequences from palms and the ends of the intron were readily identified. None of the sequences reached the 5′ end of the intron, as the 3′ end of the primer rpsF is situated 11 bases inside the intron, according to Oxelman *et al.* (1997), or 9 bases inside the intron, according to the GenBank entry of Kanakari *et al.* (1992). As primer rpsR2 lies well within *rps*16 exon 2, the 3′ end of the intron could be located. Using the *Z. mays* sequence to estimate the position of the 5′ end of the intron, the mean length of the *rps*16 intron of palms was calculated to be 870 bp (see Table 1). The GC content of each sequence was low, ranging from 32 to 35% (mean = 33%). Pairwise Jukes–Cantor distances revealed a low mean distance between sequences of 0.016 (SD = 0.009, range = 0.000–0.037).

Cladistic Results

Statistics for each maximum parsimony analysis are detailed in Table 4. The g_1 statistics are all significantly different from zero ($P < 0.01$), suggesting that significant levels of nonrandom structure exist within each dataset (Hillis and Huelsenbeck, 1992).

ITS analysis 1. In the strict consensus of the trees found in ITS analysis 1 (Fig. 1), the Calamoideae are

resolved as monophyletic (jackknife = 73%). Three major clades are resolved within the Calamoideae. However, the relationships between these clades are ambiguous. The first clade comprises the genera of the Ancistrophyllinae and Oncocalaminae (*Laccosperma, Eremospatha,* and *Oncocalamus;* hereafter referred to as the African rattans) and has no jackknife support. The second major clade, also lacking jackknife support, contains the genera of the Asian subtribe Eugeissoninae, the African subtribe Raphiinae, and the New World tribe Lepidocaryeae. The Lepidocaryeae is resolved as a moderately supported monophyletic group (jackknife = 71%) sister to *Raphia,* and *Eugeissona* is sister to the *Raphia*–Lepidocaryeae clade. Thus, tribe Calameae is resolved as a paraphyletic group, although there is no jackknife support for these relationships.

The third major clade also lacks jackknife support. It is almost exclusively Asian (except for *Calamus deerratus* from Africa) and contains the genera of the Metroxylinae, Calaminae, Plectocomiinae, and Pigafettinae. This clade is hereafter referred to as the Asian clade. Subtribes Calaminae and Metroxylinae are resolved as nonmonophyletic. There is a basal polytomy within the clade which supports three monophyletic groups. The first of these includes *Salacca* and *Eleiodoxa* from the Calaminae (hereafter termed the *Salacca* clade) and is poorly supported (jackknife = 57%). The second clade contains only *Korthalsia* of the Metroxylinae (jackknife = 99%). The third clade on the polytomy comprises the rattan genera of the Calaminae (*Calamus, Daemonorops, Calospatha, Pogonotium, Ceratolobus,* and *Retispatha*), subtribes Plectocomiinae and Pigafettinae, and *Metroxylon* of the Metroxylinae. A monophyletic Plectocomiinae is included with the rattans of the Calaminae, within which relationships are largely ambiguous. *Pigafetta* is sister to this clade and *Metroxylon* is sister to the *Pigafetta*–Plectocomiinae–rattans of the Calaminae clade. Again, there is no jackknife support for these relationships.

Throughout the tree, there are instances in which clones from individuals do not form monophyletic groups (e.g., *Laccosperma opacum, L. acutiflorum, Eugeissona tristis, Mauritia flexuosa, Calamus castaneus, Retis-*

TABLE 4

Statistics Calculated from Maximum Parsimony Analyses of ITS and *rps*16 Intron Sequences

Analysis	Number of taxa	Informative characters	Tree length	Tree number	CI	RI	RC	CI$_{9}$′	S (ss)	g_1
ITS analysis 1	108	469	3941	405	0.23	0.62	0.15	0.84	0.53	−0.50
ITS analysis 2	60	415	3125	32	0.25	0.39	0.10	0.76	0.63	−0.34
ITS analysis 3	60	415	263039	1	0.46	0.72	0.33	—	—	—
*rps*16 analysis 1	41	44	59	5	0.83	0.94	0.78	0.97	0.59	−0.63
Simultaneous analysis 1	38	411	2105	10	0.34	0.42	0.14	0.75	0.86	−0.49
Simultaneous analysis 2	38	411	269529	1	0.61	0.78	0.48	—	—	—

patha dumetosa, Pogonotium ursinum, Daemonorops fissa, and *D. didymophylla*). The implications of this phenomenon are addressed below.

ITS analysis 2. Two islands of equally most-parsimonious trees were discovered in ITS analysis 2. A strict consensus tree with jackknife values and one of the fundamental trees for ITS analysis 2 are shown in Figs. 2 and 3, respectively. The topology of the strict consensus is similar to that found in ITS analysis 1 and there are equally low levels of jackknife support. The African rattans form a monophyletic group with the Lepidocaryeae, *Raphia,* and *Eugeissona.* The African rattans are not monophyletic, as *Eremospatha* resolves as sister to *Raphia.* The topology of the Asian clade is congruent with that found in ITS analysis 1, although a clade containing *Korthalsia, Salacca,* and *Eleiodoxa* is resolved but not supported by the jackknife. The rattan genera of the Calaminae are resolved as monophyletic but the relationships of this group (hereafter termed the *Calamus* clade) with the Plectocomiinae, *Pigafetta,* and *Metroxylon* are ambiguous.

ITS analysis 3. A stable topology was obtained after only two rounds of successive approximations weighting. The tree (Fig. 4) is largely congruent with ITS analyses 1 and 2 but is better supported after successive weighting. For example, the monophyly of the Calamoideae was supported with a jackknife value of 94% in the successively weighted analysis, as compared with 74% under the equal weights of analysis 2. A clade containing the African rattans is well supported (jackknife = 92%) and is sister to an unsupported clade containing all remaining Calamoideae. The Lepidocaryeae–*Raphia*–*Eugeissona* clade is resolved but not supported and is sister to all remaining Calamoideae, except the African rattans. The Lepidocaryeae clade is very highly supported (jackknife = 99%). The Asian clade is quite well supported (jackknife = 77%) and includes a very well-supported *Salacca* clade (jackknife = 95%) which is sister to all remaining members of the clade. The remainder of the Calaminae forms a well-supported clade (jackknife = 88%) with the Plectocomiinae. *Pigafetta* is sister to this group. The Metroxylinae is resolved as monophyletic, although it is not supported by the jackknife, and is sister to the *Pigafetta*–*Plectocomiinae*–*Calamus* clade.

ITS analysis 4. The analysis was stopped after 100 h had passed and 38,000 rearrangements had been tried. A single tree of −ln likelihood 13962.49361 was found. The topology is moderately congruent with that of the previous analyses. The Lepidocaryeae–*Raphia* clade is resolved as sister to all remaining Calamoideae. *Eugeissona,* which is resolved with the Lepidocaryeae–*Raphia* clade in all previous analyses, forms a clade with a monophyletic African rattan group. This *Eugeissona*–African rattan clade is sister to the Asian

clade. The topology within the Asian clade includes the familiar group comprising the Plectocomiinae and the *Calamus* clades, both of which are monophyletic. This clade is sister to a monophyletic group containing *Metroxylon* and *Pigafetta.* A *Metroxylon*–*Pigafetta* clade does not appear in any strict consensus from other ITS analyses. *Korthalsia* is sister to all other members of the Asian clade, and the *Salacca* clade resolves as sister to all members of the Asian clade excluding *Korthalsia.*

rps16 analysis 1. A strict consensus tree with jackknife values is illustrated in Fig. 5. The topology of the strict consensus shows a high degree of congruence with the topologies derived from the ITS dataset. The basal node in the Calamoideae is a polytomy. This polytomy exists in all fundamental trees, which indicates that it can be attributed to a lack of data rather than conflict between fundamental trees. Three clades arise from the polytomy. The first clade contains only *Eugeissona,* a genus which was resolved with either the African rattans or the Lepidocaryeae–*Raphia* clade in the ITS analyses. The second clade comprises African and American taxa (the African rattans, the Lepidocaryeae, *Raphia*) and is weakly supported (jackknife = 56%). Within this clade, the African rattan clade is well supported (jackknife = 100%) and is resolved as sister to the Lepidocaryeae–*Raphia* clade which is poorly supported (jackknife = 56%). The Lepidocaryeae, although monophyletic, are only weakly supported (jackknife = 59%). The third clade is equivalent to the Asian clade discussed above and is well supported (jackknife = 81%) but contains several polytomies due to lack of data. Within the Asian clade, *Korthalsia* is resolved as sister to a well-supported group (jackknife = 81%) comprising all remaining members of the Asian clade. The *Calamus* clade is resolved and moderately supported (jackknife = 62%) but the previously recovered simple relationship with the Plectocomiinae is not resolved. Rather, the *Calamus* clade shares a polytomy with *Pigafetta* and *Myrialepis.* The remaining members of the Plectocomiinae are resolved as a monophyletic group on a polytomous node at which the *Salacca* clade and *Metroxylon* also resolve.

rps16 analysis 2. After the first round of successive approximations weighting, a topology identical to the strict consensus of analysis 1 was recovered, a finding explained by the low level of character conflict in the dataset. Thus, successive weighting of the *rps*16 dataset will not be considered further here.

rps16 analysis 3. After 50 h and 39,000 rearrangements, the analysis was stopped. A total of 3767 trees of −ln likelihood 2029.85163 were saved. The strict consensus of all 3767 fundamental trees was identical to that obtained in *rps*16 analysis 1 (Fig. 5). The additional resolution that appears in the fundamental trees
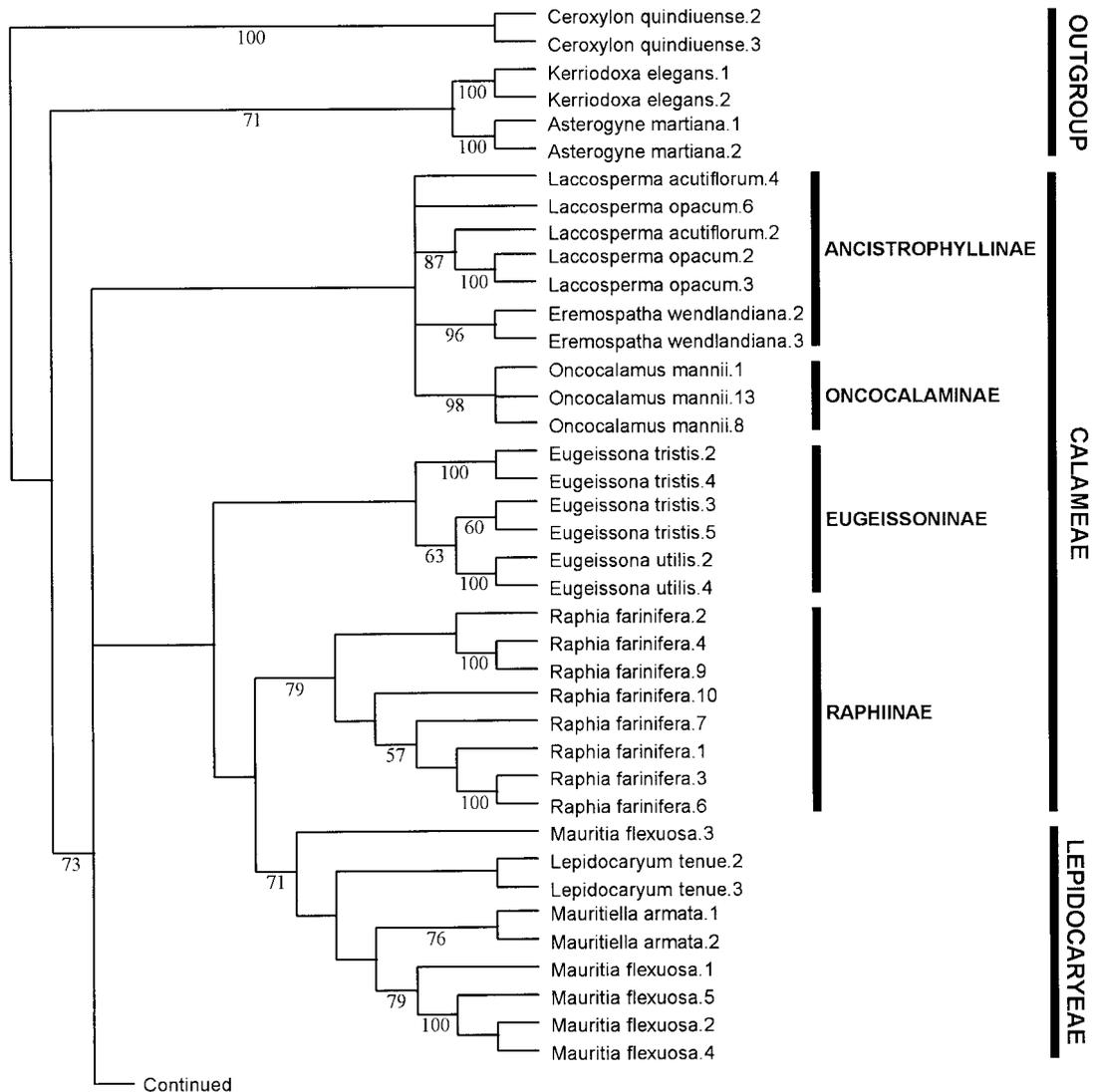
FIG. 1. Strict consensus of 405 equally most-parsimonious trees found during ITS analysis 1. Numbers below branches indicate jackknife support values.

can be attributed to extremely short branches (instantaneous probability of substitution per site = $1 \times e^{-8}$). Although statistics have not been computed, it is unlikely that these branches differ significantly in length from zero.

Simultaneous analysis 1. Three islands of equally most-parsimonious trees were discovered in simultaneous analysis 1. The strict consensus tree (Fig. 6) is moderately resolved but possesses relatively few nodes supported by the jackknife. The Calamoideae is resolved as monophyletic with high jackknife support (jackknife = 98%). Within the Calamoideae, four major clades are resolved, the first two with jackknife support (*Eugeissona,* jackknife = 100%; African rattan clade = 76%) and the second two without support (*Raphia*–Lepidocaryeae clade, Asian clade). A clade comprising all Calamoideae except *Eugeissona* is re-

solved, making *Eugeissona* sister to all remaining members of the subfamily, but this relationship is not supported. Equally, a sister group relationship between the *Raphia*–Lepidocaryeae clade and the Asian clade lacks jackknife support. The Lepidocaryeae is well supported (jackknife = 93%) but relationships between major groups in the Asian clade are largely unresolved or unsupported. *Metroxylon* is placed in a sister position to all remaining members of the Asian clade but this relationship is not supported by the jackknife. Within the Asian clade, the *Salacca* clade and the Plectocomiinae are resolved with jackknife support of 80 and 61%, respectively, but the *Calamus* clade, though resolved, is unsupported.

Simultaneous analysis 2. After two rounds of successive approximations weighting, a stable topology was recovered. The tree (Fig. 7) comprises three major
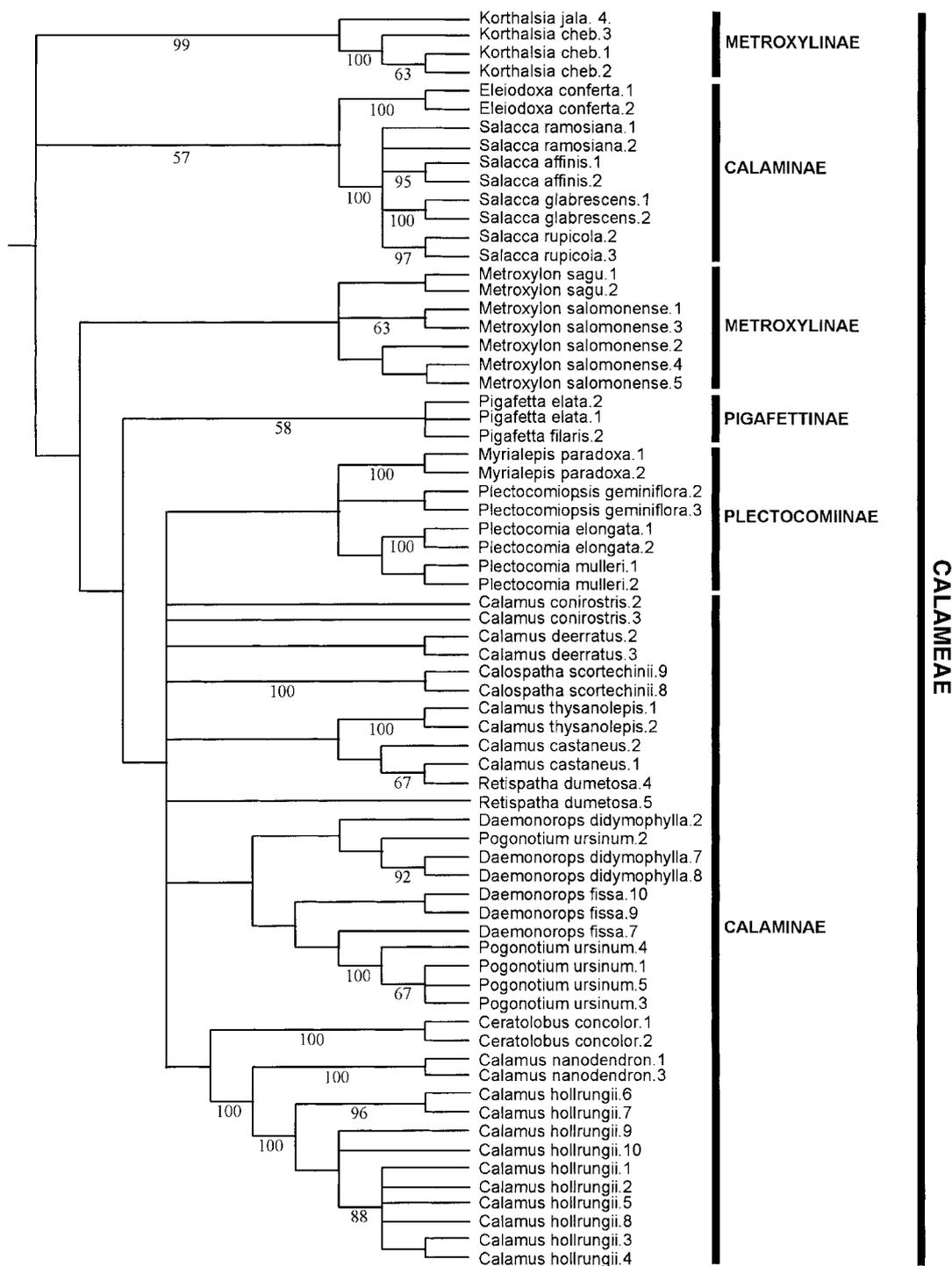
FIG. 1—*Continued*

clades, the first of which contains only *Eugeissona* and is highly supported (jackknife = 100%). The second clade contains all African and American taxa (the African rattans, *Raphia,* the Lepidocaryeae) and has weak jackknife support of 62%. Within this African–American clade, the Lepidocaryeae is highly supported (jackknife = 100%) and its sister group relationship to

Raphia is moderately supported (jackknife = 74%). The African rattans also form a robust group within the African–American clade (jackknife = 100%).

The Asian clade, the third major group resolved within the tree, is very highly supported with a jackknife value of 96%. However, its position as sister to the African–American clade is not supported. Within the
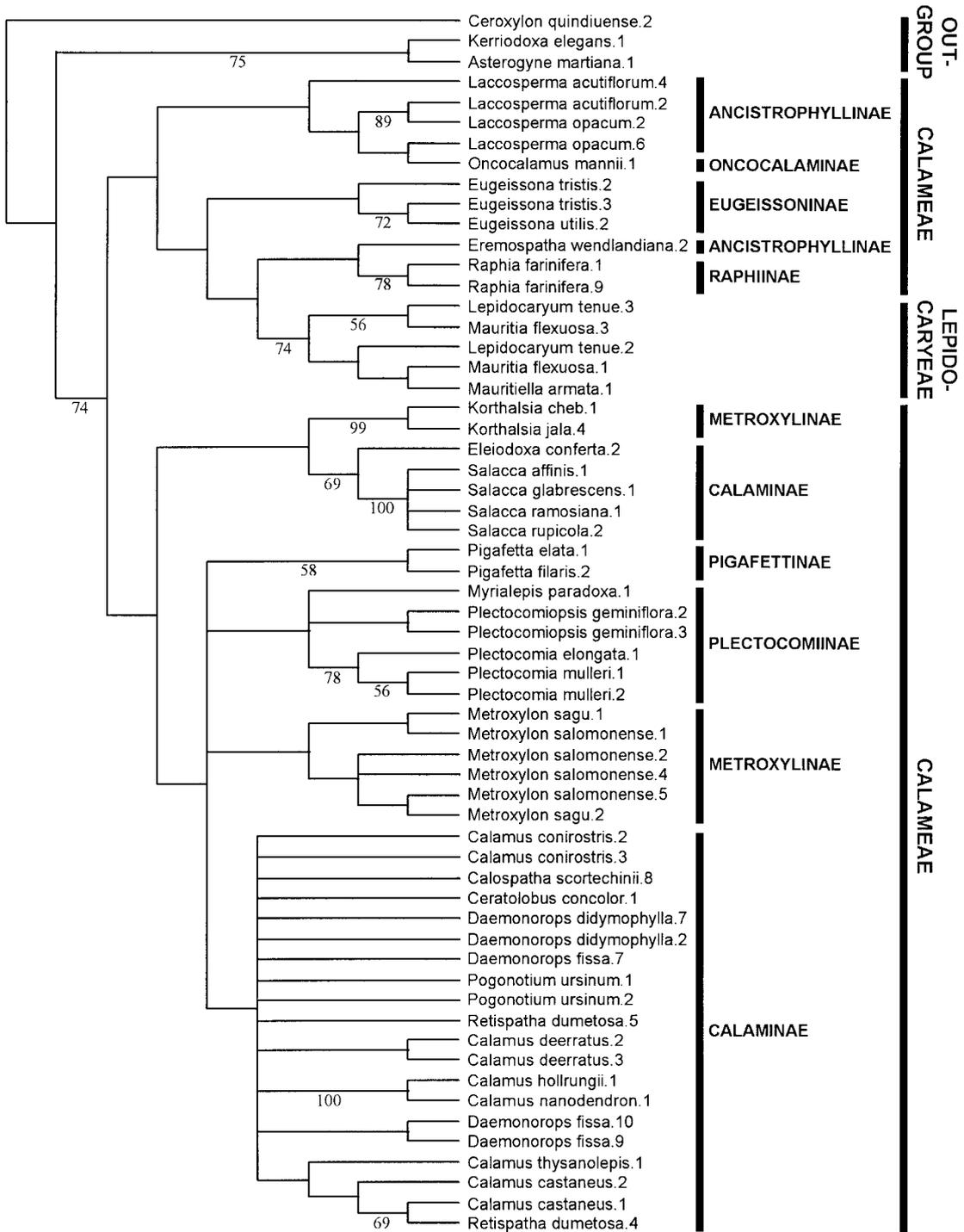
FIG. 2. Strict consensus of 32 equally most-parsimonious trees found during ITS analysis 2. Numbers below branches indicate jackknife support values.

Asian clade, the *Salacca* clade, the Plectocomiinae, and the *Calamus* clade are resolved, with 99, 86, and 99% jackknife support, respectively. Several significant and well-supported sister group relationships are recovered: *Korthalsia* is sister to all remaining Asian clade members, the *Salacca* clade is sister to all members except for *Korthalsia,* and the Plectocomiinae is sister to the *Calamus* clade. *Pigafetta* is resolved as the sister group of the Plectocomiinae–*Calamus* clade but this relationship lacks jackknife support.
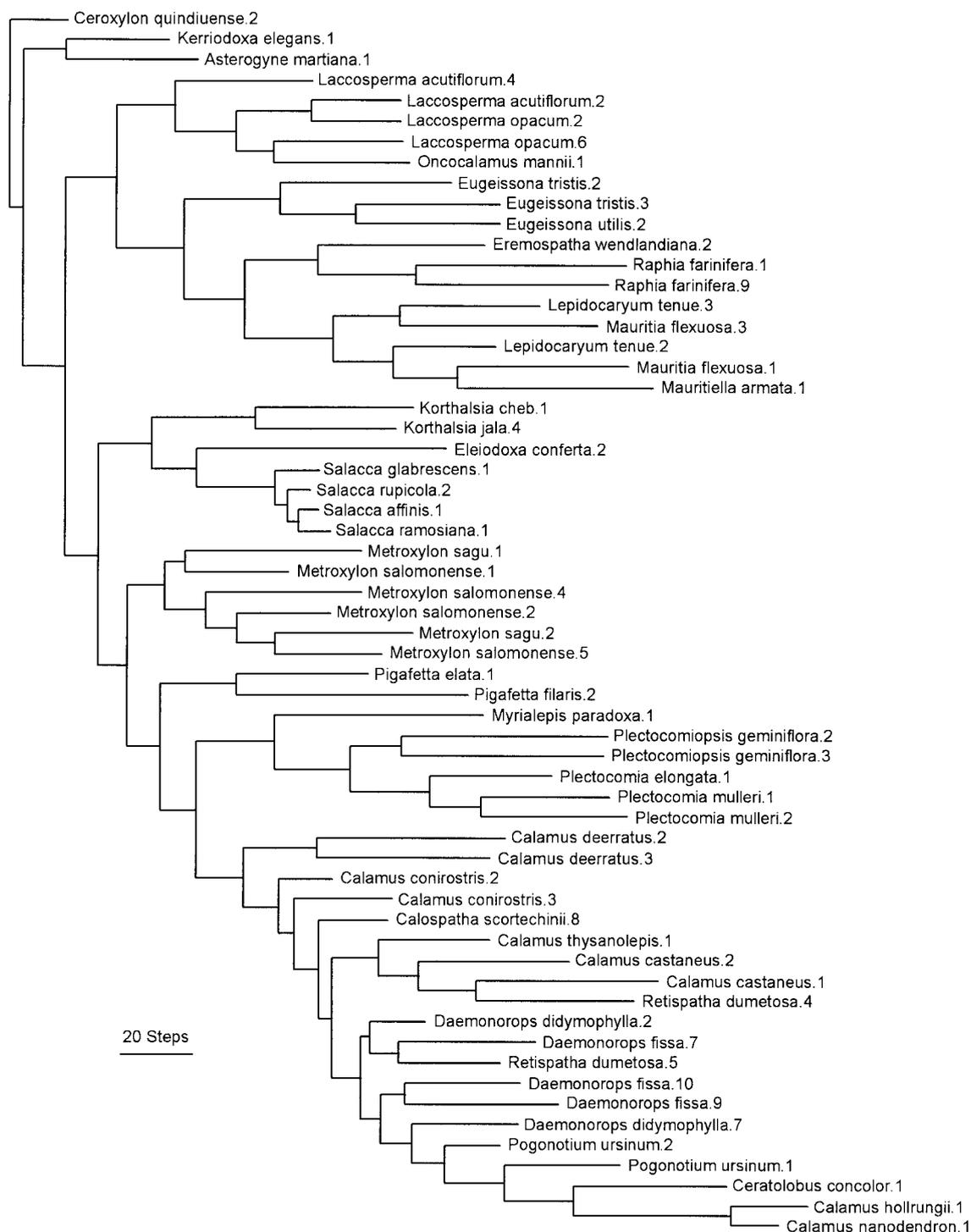
FIG. 3. One tree (length = 3125, CI = 0.25, RI = 0.39, RC = 0.10) chosen arbitrarily from 32 equally most-parsimonious trees found during ITS analysis 2.

DISCUSSION

Molecular Evolution in ITS Regions and rps16 Intron of the Calamoideae

A discussion of molecular evolution falls somewhat outside the scope of this project. However, some consid-

eration of this issue and its impact on phylogeny reconstruction is warranted in the light of the discussion of palm molecular evolution given above and the discovery of high levels of within-individual polymorphism in the ITS region of palms.

As substitution rates have not been calculated in this
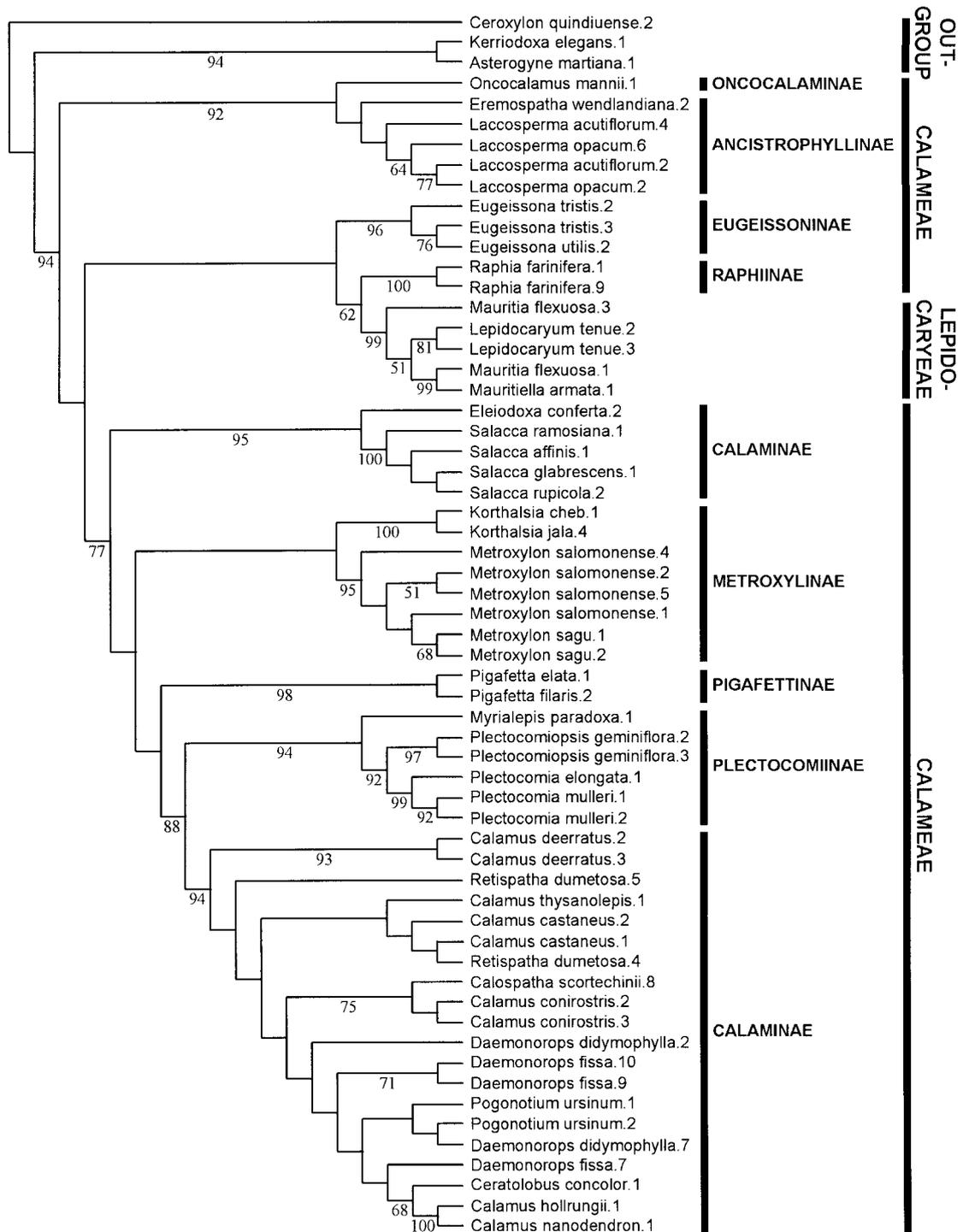
FIG. 4. Single tree found during ITS analysis 3 (length = 263039, CI = 0.46, RI = 0.72, RC = 0.33). Numbers below branches indicate jackknife support values.

study, a direct comparison with the general finding that palm DNA evolves slowly (Wilson *et al.,* 1990; Gaut *et al.,* 1992, 1996; Eyre-Walker and Gaut, 1997) is not possible. However, pairwise Jukes–Cantor distances between the three grass *rps*16 sequences available in GenBank in October 1997 have been calculated. These

can be compared with those of the palms to give an estimate of relative divergence, irrespective of time. The *rps*16 intron sequences of *Hordeum vulgare* (Gen-Bank Accession Nos. X52765, X54320), *Oryza sativa* (GenBank Accession No. X15901), and *Zea mays* (Gen-Bank Accession No. X60823) were aligned and their
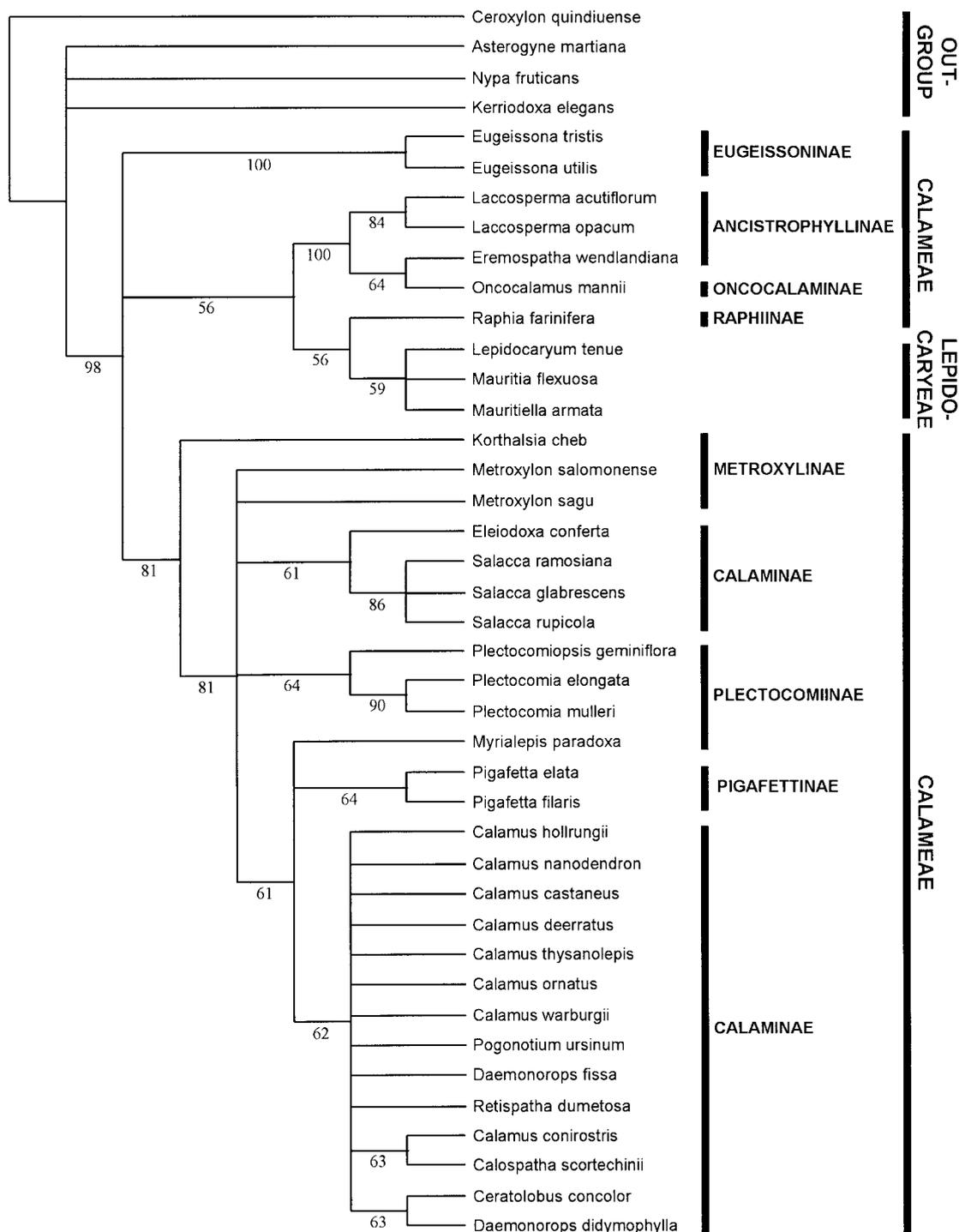
FIG. 5. Strict consensus of five equally most-parsimonious trees found during *rps*16 analysis 1. Numbers below branches indicate jackknife support values.

pairwise Jukes–Cantor distances calculated using PAUP*. The average distance was 0.067 (*Hordeum* vs *Oryza* = 0.073, *Hordeum* vs *Zea* = 0.075, *Oryza* vs *Zea* = 0.054), over four times the average distance between sequences in the palm dataset. The average distance for palms of 0.016 may be artificially low,

as ambiguously aligned regions in the palm dataset were excluded from the calculation of distances. However, this evidence is consistent with lower levels of divergence in the *rps*16 intron of palms than that of grasses, a finding which provides circumstantial evidence in support of the generalization that chloroplast
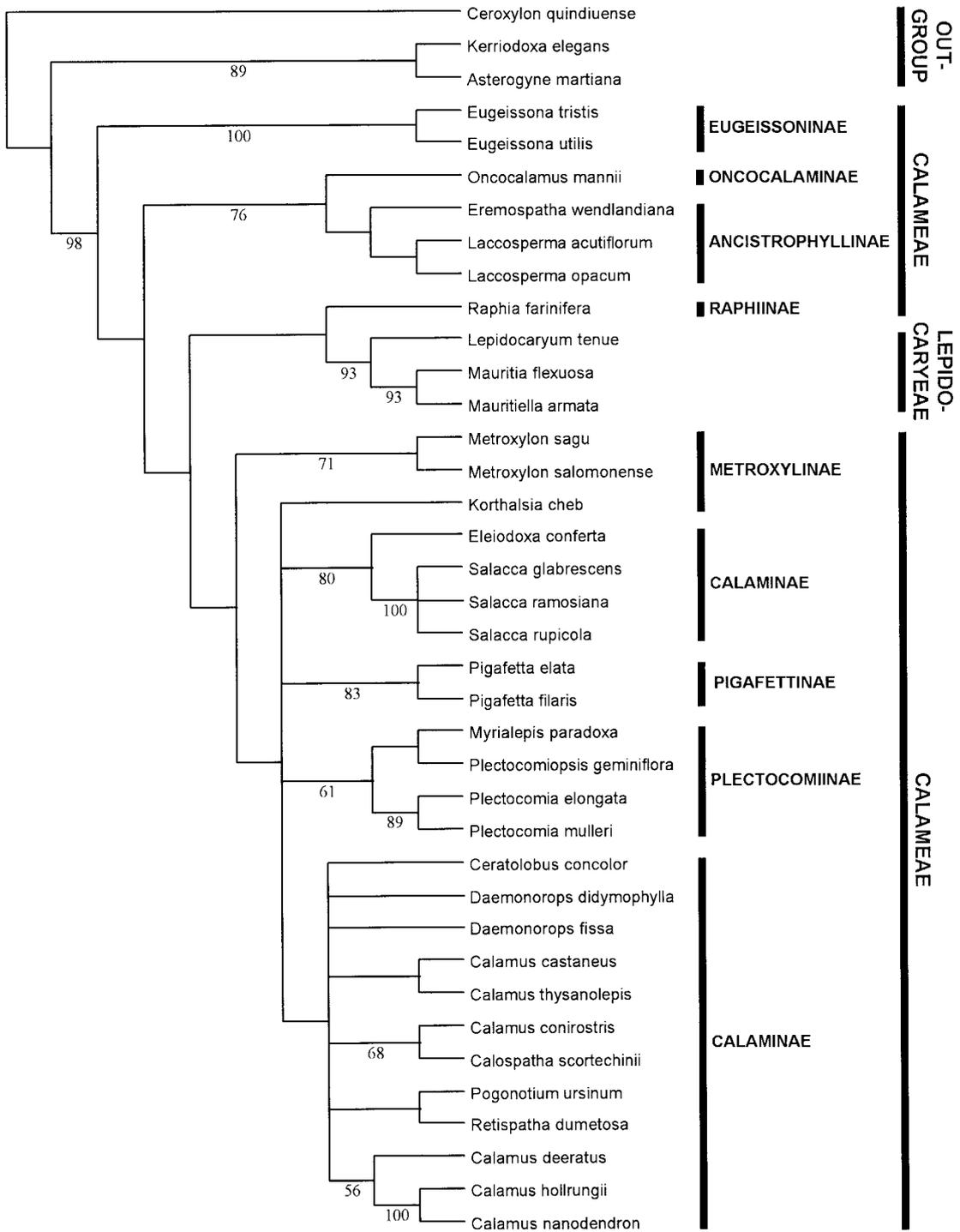
FIG. 6. Strict consensus of 10 equally most-parsimonious trees found during simultaneous analysis 1. Numbers below branches indicate jackknife support values.

DNA evolves slowly in the palm family (Wilson *et al.,* 1990).

The range of intergenome Jukes–Cantor pairwise distances calculated for the entire ITS region of palms (0.015 (*Salacca affinis.* 1 vs *Salacca ramosiana.* 1) to 0.404 (*Calamus hollrungii.* 8 vs *Mauritia flexuosa.* 4,

Calamus hollrungii. 8 vs *Mauritia flexuosa.* 2)) over-laps broadly with the ranges of percentage pairwise distances for separate ITS1 and ITS2 regions of various angiosperm groups listed by Baldwin *et al.* (1995). Thus, the divergence of palm ITS falls well within the range known previously for closely related angiosperm
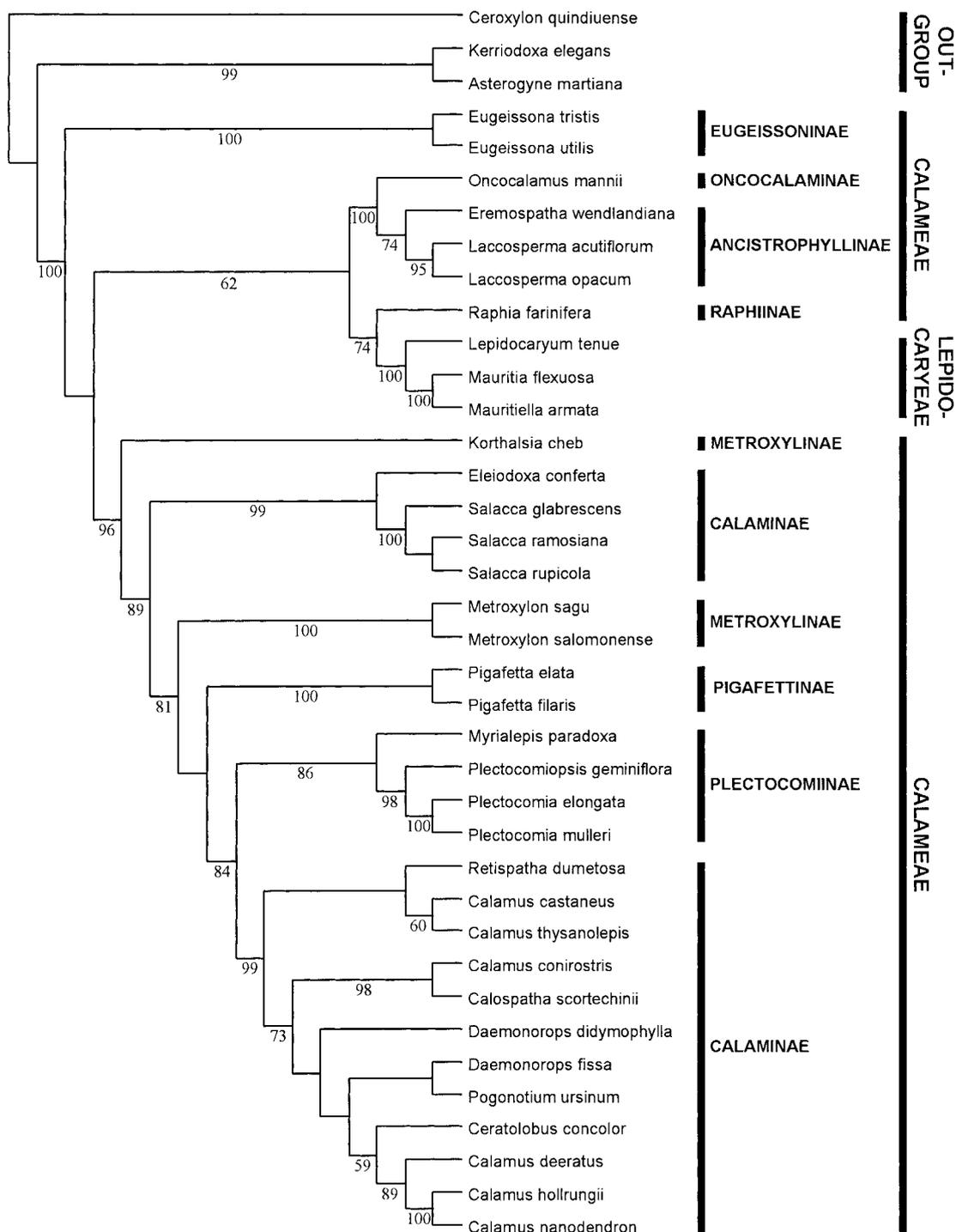
FIG. 7. Single tree found during simultaneous analysis 2 (length = 269529, CI = 0.61, RI = 0.78, RC = 0.48). Numbers below branches indicate jackknife support values.

groups, although the maximum value for palms exceeds the maximum recorded by Baldwin *et al.* (39.0% pairwise sequence divergence for ITS1 region of the Polemoniaceae). Given the inclusion of conserved regions in our calculation (5.8S, partial 18S), the maximum value for palm ITS1 might be expected to exceed 0.404. These results are not highly relevant to the finding of Gaut *et al.* (1996) that synonymous substitution rates of a protein-coding nuclear gene are lower in palms than grasses because none of the ITS region is protein coding and therefore synonymous substitution rates cannot be calculated.

The current study has revealed a remarkably high level of intragenome polymorphism in the ITS region of palms. Pairwise Jukes–Cantor distances of up to 0.258 have been calculated between clones isolated from individuals, a value that approaches the average intergenome pairwise distance of 0.261. Clearly, the ITS region in palms is not completely homogenized by the processes of concerted evolution. In the analysis of all clones in ITS analysis 1, multiple clones from most individuals are resolved as monophyletic groups, thereby presenting little problem for phylogenetic inference. However, several instances of nonmonophyletic groups of intragenomic clones are observed (e.g., Fig. 1, *Laccosperma opacum, L. acutiflorum, Eugeissona tristis, Mauritia flexuosa, Calamus castaneus, Retispatha dumetosa, Pogonotium ursinum, Daemonorops fissa, D. didymophylla*), further indicating the relaxation of concerted evolution. However, complete orthology/paralogy relationships (*sensu* Sanderson and Doyle, 1992) are not apparent. Having conducted simulation studies, Sanderson and Doyle (1992) suggested that trees similar but not identical to the "correct" orthology/paralogy tree or concerted evolution tree could be recovered at intermediate levels of concerted evolution. However, in such cases, homoplasy and tree number is high, and tree support is low. In the current study, high levels of homoplasy were observed, evidenced by low consistency indices and long terminal branch lengths (Fig. 3). Moderately large numbers of trees were found and jackknife support was generally poor, particularly so at nodes toward the base of the tree. It seems likely that, in this case, concerted evolution is operating at a level which can bring about unreliable estimates of organismal phylogeny and, therefore, the trees should be interpreted with caution.

The intragenome pairwise distances of taxa for which five or more clones were sequenced (Table 3) show that levels of intragenome polymorphism in palm ITS vary considerably between species. *Calamus hollrungii,* for example, shows consistently low levels of divergence between clones, whereas *Metroxylon salomonense* shows consistently high levels of divergence. Some clones from *Raphia farinifera* are very similar to each other, whereas others are highly divergent. Divergent paralogues have been documented in the ITS regions of a number of angiosperm groups (Buckler and Holtsford, 1996a,b; Buckler *et al.,* 1997). It has been suggested that some divergent paralogues may be pseudogenes and can be identified as such by their rarity, basal position in rooted trees, relatively low GC content, and low-stability secondary structure (Buckler *et al.,* 1997). It is not clear whether or not any of the clones in this study could be classified as pseudogenes. However, the functionality of certain clones with major length variation may be questioned. For example, *Oncocalamus mannii* clone 1 contains a 5.8S gene of only 126 bp in length, as compared to the otherwise quite conserved

average length of 163 bp. A transcript of this 5.8S copy may no longer be able to form a secondary structure with functional capacity. However, such length variation may also be an artifact of the cloning procedure. Large divergences between paralogues may also be explained by a lack of interlocus concerted evolution (cf. Wendel *et al.,* 1995; Cronn *et al.,* 1996), by introgression, or by recombination events (Buckler *et al.,* 1997).

Congruence between Different Trees from the Same Dataset

Trees from the four different analyses of the ITS dataset share many monophyletic groups: the African rattans (Ancistrophyllinae and Oncocalaminae), the Lepidocaryeae, the Lepidocaryeae–*Raphia* clade, the Asian clade (excluding *Eugeissona*), the *Salacca* clade, the Plectocomiinae, and the Plectocomiinae–*Calamus* clade. The *Calamus* clade is resolved in strict consensus trees from all analyses except ITS analysis 1.

ITS analysis 2 yielded some interesting results. For example, the sister group relationship between *Raphia* and *Eremospatha* and the consequent nonmonophyly of the African rattan clade is surprising, given that the African rattans are resolved as monophyletic in all other analyses and are intuitively a natural grouping. There is also a lack of resolution in the Asian clade. This may be an example of the way in which undersampling of paralogues from multigene families can influence gene tree topologies. It is a potentially serious concern in cases such as this in which high levels of intragenome divergence result in considerable homoplasy, and spurious organismal relationships may be resolved by means of long branch attraction. However, ITS analysis 2 included fewer terminal taxa than analysis 1 and, consequently, there is a higher chance of recovering the shortest tree. Analysis 2 must therefore be considered more rigorous from the perspective of search intensity than analysis 1.

There are several areas of conflict between the alternative topologies derived from the ITS dataset. ITS analysis 2 resolves a monophyletic group comprising the African rattans, the Lepidocaryeae, and *Raphia* and *Eugeissona* as sister to the Asian clade, whereas analysis 3 resolves the African rattans as sister to all remaining Calamoideae. Analysis 4 provides a further hypothesis, that the Lepidocaryeae–*Raphia* clade is sister to all remaining Calamoideae, and ITS analysis 1 is largely ambiguous in this region. Closer examination of the trees reveals that this incongruence can be attributed to alternative rootings into essentially similar ingroup topologies. Within the Asian clade, the topology of the grade of taxa with catkin-like rachillae (*Korthalsia, Metroxylon, Eleiodoxa, Salacca,* and *Pigafetta*) is different in each analysis, although jackknife support is lacking for every alternative arrangement.

The decision regarding which ITS analysis should be

preferred over the others is awkward, as each has its advantages and disadvantages. The dataset of analysis 1 included such a large number of terminal taxa that it was hard to efficiently analyze and it was impossible to be confident that the shortest tree had been found. The smaller dataset used in the more rigorous analysis 2 allowed a more exhaustive method of analysis to be used but the scaled consistency index, CI_9', is lower than that calculated for analysis 1, indicating a poorer fit of characters on the shortest trees. However, the maximum probability of phylogenetic inference, S, is higher than that of analysis 1 but this can probably be attributed largely to the reduction in sample size. In analysis 2, at least 94 additional local optima were found within 11 steps of the shortest tree length, indicating poor data decisiveness (Goloboff, 1991). Thus, an increase in tree length of less than 0.4% might have a profound effect on topology. The problem is also likely to be associated with the larger dataset used for analysis 1, although the search strategy employed did not allow the counting of local optima.

ITS analysis 3 involves a weighting scheme which might be perceived as a set of undesirable *ad hoc* assumptions. However, a so-called unweighted analysis, such as that of analysis 1 or 2, does incorporate weights, albeit equal ones, which are strong assumptions in themselves. The character weights used in analysis 3 are proportional to the fit of each character on an initial tree or set of trees, in this case, the trees from analysis 2 (Farris, 1969, 1989; Goloboff, 1993). Thus, the weighting scheme is derived explicitly from the data in hand and aims to minimize the impact of homoplasious characters on subsequent topologies. Although successive approximations weighting can result in the identification of local rather than global optima (Swofford *et al.*, 1996), the method is a useful solution to problematic datasets such as the ITS dataset in which a means of accounting for high homoplasy is needed. Analysis 4 provides a hypothesis based on a model of molecular evolution that accounts for differential substitution rates and rate heterogeneity between sites and thereby accommodates potential causes of homoplasy. Again, these assumptions might be considered unacceptable. Furthermore, the computation required by maximum likelihood analyses is so intensive that it proved impossible for a single heuristic search replicate to be completed. Clearly, the four analyses of the ITS dataset must be considered on their own merits, as each embodies different assumptions and pitfalls.

The alternative topologies derived from the analyses of the *rps*16 dataset are entirely congruent with each other. The additional resolution observed in the fundamental trees from the maximum likelihood analysis of the *rps*16 dataset is a result of branches with extremely low probabilities of substitution per site, which are not resolved under parsimony, as they lack character sup-

port and would therefore be collapsed, as their maximum length equals zero.

There is relatively little conflict between the strict consensus tree derived from simultaneous analyses 1 and the single tree from simultaneous analysis 2. Two significant conflicts exist. First, the *Raphia*–Lepidocaryeae clade is sister to the Asian clade in simultaneous analysis 1, whereas it is sister to the African rattan clade in simultaneous analysis 2. Second, *Metroxylon* is sister to all remaining members of the Asian clade in simultaneous analysis 1, whereas *Korthalsia* resolves in that position in simultaneous analysis 2. However, the relationships of these groups are not supported by the jackknife in simultaneous analysis 1, whereas they are supported in simultaneous analysis 2. In fact, the tree from simultaneous analysis 2 is much less ambiguous than the consensus tree from simultaneous analysis 1 and contains almost twice as many nodes supported by the jackknife. Therefore, simultaneous analysis 2 has produced a topology superior to that of analysis 1, although the same caveats described above apply with regard to the weaknesses of successive approximations weighting.

Congruence between Trees from Different Datasets

There is a high degree of congruence between the relationships recovered from the ITS dataset and the *rps*16 intron dataset. All trees include the African rattan clade, the Lepidocaryeae–*Raphia* clade, and the Asian clade (excluding *Eugeissona*). One area of incongruence can be observed in the relationships among *Eugeissona,* the African rattans, and the Lepidocaryeae–*Raphia* clade. In all ITS trees, *Eugeissona* is sister to either the African rattans or the Lepidocaryeae–*Raphia* clade. However, in the *rps*16 intron trees, the African rattans resolve as sister to the Lepidocaryeae–*Raphia* clade in an African–American clade with some jackknife support (jackknife = 56%) and *Eugeissona* resolves on the polytomous basal node in the Calamoideae shared with the African–American clade and the Asian clade. Similar relationships resolve in simultaneous analysis 2, although in this case, *Eugeissona* is resolved as sister to all other Calamoideae. The position of *Eugeissona* is clearly ambiguous.

A second area of incongruence between the relationships derived from the ITS and the *rps*16 intron datasets can be found among the Plectocomiinae, the Pigafettinae, and the rattan genera of the Calaminae. All ITS trees are congruent with a sister group relationship between the Plectocomiinae and the *Calamus* clade. The Plectocomiinae is monophyletic in all ITS trees. However, in the *rps*16 intron trees, *Myrialepis* is resolved on a polytomy with *Pigafetta* and the *Calamus* clade, whereas *Plectocomia* and *Plectocomiopsis* form a monophyletic group. This is surprising in view of the high degree of morphological similarity between *Plectocomiopsis* and *Myrialepis.* Furthermore, the mono-

phyly of the Plectocomiinae, the *Calamus* clade, and the sister group relationship of the two are very well supported in the successively weighted analysis of ITS analysis 3. The relationships described for the *rps*16 trees have only moderate jackknife support and character support for the *Myrialepis–Pigafetta–Calamus* clade is extremely low (1 nucleotide change).

Ultimately, the results of the simultaneous analyses must be considered the best molecular phylogenetic hypotheses of relationship among the Calamoideae because they are based on all available DNA sequence data. If any systematic conclusions are to be drawn on molecular evidence alone, they should be based on the simultaneous analyses. Of the two analyses, that which employed successive approximations weighting methods is favored because it yielded only one, completely resolved tree with many highly supported nodes. The most significant inadequacy of this tree is the lack of jackknife support at the node uniting the Asian clade with the African–American clade. A schematic tree which summarizes relationships that can be drawn with confidence from the simultaneous analyses is depicted in Fig. 8.

Congruence with Trees from Morphology

The molecular phylogenies generated in this study are not very congruent with morphological phylogenies. The outcome of the morphological analyses of Baker *et al.* (1999a) bears little resemblance to that from any molecular analyses. Common monophyletic groups include the Lepidocaryeae, the *Salacca* clade, the Plectocomiinae, the *Calamus* clade, and the Plectocomiinae–*Calamus* clade.

Similarly, there is little congruence between the molecular phylogenies and that of McClatchey (1996), except that the monophyly of the African rattans and the Lepidocaryeae is resolved in both.

General Implications for Morphology and Classification

A number of monophyletic groups emerge consistently from the analysis of molecular data: the African rattans, the Lepidocaryeae–*Raphia* clade, and the Asian clade (excluding *Eugeissona*). Unfortunately, the relationships of *Eugeissona* are ambiguous and its position is not supported in any analysis but in all instances this morphologically distinct genus resolves outside the three major groups mentioned above and it is best considered as a separate lineage. The African–American clade resolves only in analyses of *rps*16 intron data and in simultaneous analysis 2 and is weakly supported in both. A similar group which includes *Eugeissona* as well as the African–American taxa appears in the strict consensus tree from ITS analysis 2 but jackknife support is lacking. The African–American groupings will not be considered further here.

The African rattan clade is very well defined morpho-
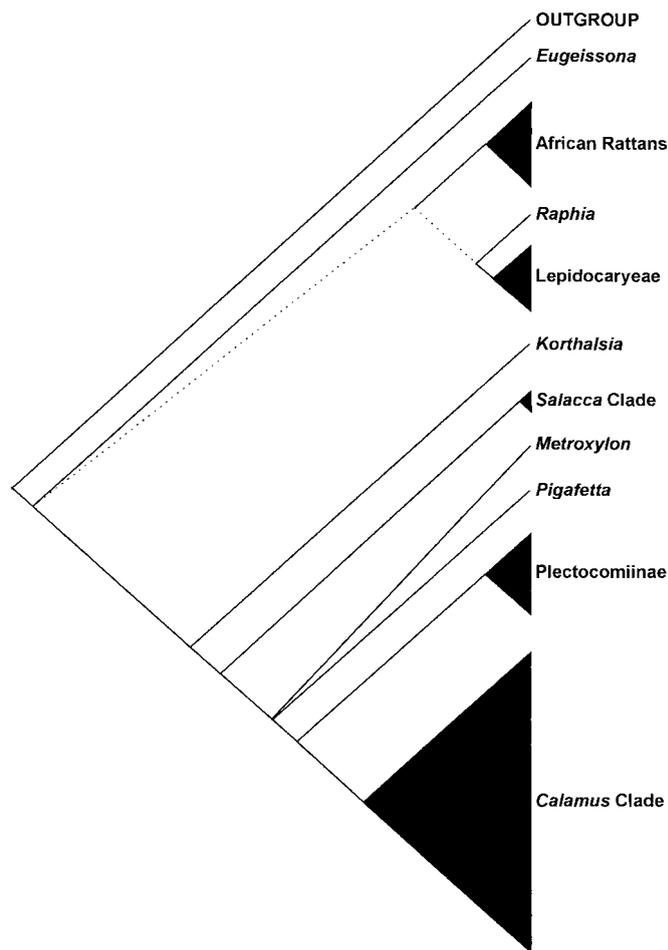
FIG. 8. Schematic tree summarizing general conclusions that can be drawn from simultaneous analysis 2. Dotted branches indicate relationships that are supported by jackknife values of less than 80%. Branches that lack jackknife support have been collapsed. (African rattans = *Laccosperma*, *Eremospatha*, and *Oncocalamus*; *Salacca* clade = *Salacca* and *Eleiodoxa*; *Calamus* clade = *Calamus*, *Daemonorops*, *Calospatha*, *Ceratolobus*, *Pogonotium*, and *Retispatha*.)

logically, with its distinctive climbing organ, the cirrus bearing acanthophylls, and its inflorescences branched to one order. Although there is some disagreement among the topologies of the various analyses, simultaneous analysis 2 very strongly supports the monophyly of the Ancistrophyllinae. While Uhl and Dransfield (1987) acknowledged the similarity between the members of the Ancistrophyllinae and the Oncocalaminae, this was not reflected in the positioning of the two subtribes in their classification of the Calamoideae. A revised classification might place the two subtribes next to each other or possibly include all three genera in a single subtribe.

The monophyly of the Lepidocaryeae is the least remarkable relationship resolved in this study. The morphological similarities among the genera, the palmate, reduplicate leaves, unique in the subfamily, general inflorescence structure, and the very similar

pollen strongly suggest that they are closely related. Relationships within the tribe are not resolved by *rps*16 intron data but are well resolved by ITS data. The topologies within the Lepidocaryeae from the four ITS analyses are largely congruent. Clone 3 of *Mauritia flexuosa* resolves consistently as the sister of the remaining Lepidocaryeae clones and does not form a clade with the remaining four clones from the same individual. It is possible that it may represent a pseudogene, as it appears to be rare and possesses indels that the remaining sequences do not have. In ITS analysis 2, *Lepidocaryum tenue*.3 resolves with *Mauritia flexuosa*.3. However, if these clones are disregarded, the ITS topologies suggest that *Mauritia* and *Mauritiella* are more closely related to each other than either is to *Lepidocaryum.* The close relationship of *Mauritia* and *Mauritiella* is well supported in the successively weighted analysis of ITS data alone and of the combined dataset (jackknife = 99 and 100%, respectively).

The relationship of *Raphia* to the Lepidocaryeae is a novel finding of this study. The relationship is moderately supported by simultaneous analysis 2 (jackknife = 74%). There is little morphological resemblance between *Raphia* and the Lepidocaryeae, except that both groups include but are not exclusively composed of robust tree palms, and their leaf petioles and rachises are unarmed. However, the morphological basis of this relationship requires further investigation.

The Asian clade, though very well supported in simultaneous analysis 2 (jackknife = 96%), has no conspicuous morphological basis. The one striking character in the group is the almost ubiquitous presence of equatorial and subequatorial diaperturate pollen. This type of pollen is found nowhere else in the Palmae. There are two notable exceptions within the clade: *Pigafetta* with inaperturate pollen and *Pogonotium* with uniaperturate pollen. Relationships within the clade are not entirely unambiguous at this stage. There is strong evidence that the rattan genera of the Calaminae are monophyletic (the *Calamus* clade) and that they are sister to a monophyletic Plectocomiinae, a very credible relationship, given close correspondence in climbing habit and reproductive structure. Relationships within the *Calamus* clade are not obvious at this stage and are investigated further in a subsequent paper (Baker *et al.,* 2000). However, all topologies agree with the previous finding that *Calamus* is paraphyletic (Kramadibrata, 1992). Relationships between the genera of the subtribe Plectocomiinae are rather variable across analyses but simultaneous analysis 2 suggests that *Plectocomiopsis* is more closely related to *Plectocomia* than it is to *Myrialepis,* an unexpected finding in view of the morphological similarities linking *Plectocomiopsis* and *Myrialepis.* The Asian clade taxa with catkin-like rachillae (*Korthalsia, Metroxylon, Salacca, Eleiodoxa,* and *Pigafetta*) tend to resolve as a

basal grade within the Asian clade, although the *Salacca* clade is resolved in all analyses, often with high support. Only in simultaneous analysis 2 are the relationships of the members of this basal grade well supported. The position of *Korthalsia* as sister to all remaining members of the Asian clade is apparently robust and indicates that the Metroxylinae is not monophyletic. Morphologically, *Korthalsia* can be distinguished from the other members of the Asian clade by the absence of a sarcotesta in the fruit. The *Salacca* clade too is well supported in its position as sister to all the Asian clade, except for *Korthalsia.* The relationships among *Metroxylon, Pigafetta,* and the Plectocomiinae–*Calamus* clade lack support and will be considered ambiguous at this stage.

CONCLUSIONS

While palm DNA is thought to evolve slowly, adequate levels of variation have been observed in both nuclear and chloroplast genes to give well-resolved phylogenies. DNA sequences from both genomes have provided informative and highly congruent hypotheses of relationship among the members of the Calamoideae. Concerted evolution appears to be operating at an intermediate level on the ITS region of palms, which explains the high levels of homoplasy and suggests that phylogenies derived from ITS data may not be very reliable. Indeed, there is some incongruence between the results obtained using different analytical approaches to the ITS dataset but, in general, congruence between these topologies and those from the chloroplast *rps*16 dataset is high. Furthermore, the *rps*16 trees show jackknife support for clades which appear but lack jackknife support in unweighted analyses of ITS data. Successive approximations weighting of the ITS data also provides increased support for these clades but the highest support is observed in trees obtained from successive weighting during simultaneous analysis.

Some robust systematic conclusions can be drawn. The Calameae appears to be a paraphyletic group with the Lepidocaryeae nested within it. The African genus *Raphia* is consistently resolved as sister to the Lepidocaryeae. The African rattans form a monophyletic group, as do all Asian Calamoideae except for *Eugeissona.* The relationships of the genus *Eugeissona* are still obscure. The Calaminae is not monophyletic, the six rattan genera forming a clade which is sister to a monophyletic Plectocomiinae. *Eleiodoxa* and *Salacca* form a clade in a grade of other genera with catkin-like rachillae, including the Metroxylinae, which appears not to be monophyletic, and *Pigafetta.* The apparent incongruence of these findings with morphological topologies will be addressed in a future paper in which molecular and morphological datasets will be analyzed simultaneously, morphological character evolution will

APPENDIX 1

List of Taxa Included in This Study and Their Systematic Positions (*sensu* Uhl and Dransfield, 1987), with Collection Numbers and Locations of Voucher Specimens, and EMBL Nucleotide Sequence Database Accession Nos. for Each Sequence

Tribe	Subtribe	Species	Voucher specimen	*rps*16 intron EMBL Accession No.	ITS Clone	ITS EMBL Accession No.
Calameae	Ancistrophyllinae	*Laccosperma acutiflorum* (Becc.) J. Dransf.	Dransfield JD7006 (K)	AJ241276	2	AJ242121
					4*	AJ242122
		Laccosperma opacum (G. Mann & H. Wendl.) Drude	Sunderland 1750 (K)	AJ242181	2	AJ242123
					3	AJ242124
					6*	AJ242125
		Eremospatha wendlandiana Becc.	Dransfield JD7004 (K)	AJ241277	2*	AJ242129
					3	AJ242130
	Eugeissoninae	*Eugeissona tristis* Griff.	Baker 501 (KEP)	AJ241278	2	AJ242115
					3*	AJ242116
					4	AJ242117
					5	AJ242118
		Eugeissona utilis Becc.	Baker 712 (SAR)	AJ242180	2*	AJ242119
					4	AJ242120
	Metroxylinae	*Metroxylon sagu* Rottb.	Baker 550 (SAN)	AJ242174	1*	AJ242105
					2	AJ242106
		Metroxylon salomonense (Warb.) Becc.	Zona 651 (FTG)	AJ242173	1*	AJ242107
					2	AJ242108
					3	AJ242109
					4	AJ242110
					5	AJ242111
		Korthalsia cheb Becc.	Baker 513 (K)	AJ242175	1*	AJ242101
					2	AJ242102
					3	AJ242103
		Korthalsia jala J. Dransf.	Baker 558 (K)		4	AJ242104
	Calaminae	*Eleiodoxa conferta* (Griff.) Burret	Dransfield JD6514 (K)	AJ242179	1	AJ242091
					2*	AJ242092
		Salacca affinis Griff.	Baker 708 (SAR)		1	AJ242093
		Salacca glabrescens Griff.	1984–3791 (K)	AJ242177	1*	AJ242095
					2	AJ242096
		Salacca ramosiana J.P. Mogea	1979–4409 (K)	AJ242176	1*	AJ242097
					2	AJ242098
		Salacca rupicola J. Dransf.	Baker 710 (K)	AJ242178	2*	AJ242099
					3	AJ242100
		Daemonorops fissa Blume	Baker 546 (K)	AJ242164	7	AJ242073
					9*	AJ242074
					10	AJ242075
		Daemonorops didymophylla Becc.	Baker 692 (K)	AJ242165	2*	AJ242070
					7	AJ242071
					8	AJ242072
		Calamus castaneus Griff.	Baker 507 (KEP)	AJ242155	1	AJ242046
					2*	AJ242047
		Calamus conirostris Becc.	Baker 516 (K)	AJ242156	2*	AJ242048
					3	AJ242049
		Calamus deerratus G. Mann & H. Wendl.	Tsiforkor s.n. (K)	AJ242157	2	AJ242050
					3*	AJ242051

APPENDIX 1—*Continued*

Tribe	Subtribe	Species	Voucher specimen	*rps*16 intron EMBL Accession No.	ITS	
					Clone	EMBL Accession No.
		Calamus hollrungii Becc.	Dransfield JD7571 (K)	AJ241279	1*	AJ242052
					2	AJ242053
					3	AJ242054
					4	AJ242055
					5	AJ242056
					6	AJ242057
					7	AJ242058
					8	AJ242059
					9	AJ242060
					10	AJ242061
		Calamus nanodendron J.Dransf.	Baker 720 (K)	AJ242154	1*	AJ242062
					3	AJ242063
		Calamus ornatus Blume	Dransfield JD7628 (KEP)	AJ242159		
		Calamus thysanolepis Hance	Baker & Utteridge 13 (K)	AJ242158	1*	AJ242064
					2	AJ242065
		Calamus warburgii K.Schum.	Dransfield JD7612 (K)	AJ242160		
		Calospatha scortechinii Becc.	1990–2783 (K)	AJ242161	8*	AJ242066
					9	AJ242067
		Pogonotium ursinum (Becc.) J. Dransf.	Baker 517 (K)	AJ242163	1*	AJ242076
					2	AJ242077
					3	AJ242078
					4	AJ242079
					5	AJ242080
		Ceratolobus concolor Blume	Baker 559 (K)	AJ242162	1*	AJ242068
					2	AJ242069
		Retispatha dumetosa J.Dransf.	Baker 530 (K)	AJ242166	4*	AJ242081
					5	AJ242082
	Plectocomiinae	*Myrialepis paradoxa* (Kurz) J.Dransf.	Baker 491 (KEP)	AJ242169	1*	AJ242083
					2	AJ242084
		Plectocomiopsis geminiflora (Griff.) Becc.	Baker 492 (KEP)	AJ242170	2*	AJ242089
					3	AJ242090
		Plectocomia elongata Mart. ex Blume	1984–4821 (K)	AJ242167	1*	AJ242085
					2	AJ242086
		Plectocomia mulleri Blume	Baker 563 (K)	AJ242168	1*	AJ242087
					2	AJ242088
	Pigafettinae	*Pigafetta elata* (Mart.) H.Wendl.	Baker 508 (K)	AJ242171	1*	AJ242112
					2	AJ242113
		Pigafetta filaris (Giseke) Becc.	Dransfield JD7610 (K)	AJ242172	2*	AJ242114
	Raphiinae	*Raphia farinifera* (Gaertn.) Hyl.	Rutherford 156 (K)	AJ242184	1*	AJ242131
					2	AJ242132
					3	AJ242133
					4	AJ242134
					6	AJ242135
					7	AJ242136
					9	AJ242137
					10	AJ242138
	Oncocalaminae	*Oncocalamus mannii* (H. Wendl.) H. Wendl. & Drude	Sunderland 1759 (K)	AJ241376	1*	AJ242126
					8	AJ242127
					13	AJ242128

Tribe	Subtribe	Species	Voucher specimen	*rps*16 intron EMBL Accession No.	ITS	
					Clone	EMBL Accession No.
Lepidocaryeae		*Mauritia flexuosa* L.f.	Ely *et al.* 17 (K)	AJ241281	1*	AJ242141
					2	AJ242142
					3	AJ242143
					4	AJ242144
					5	AJ242145
		Mauritiella armata (Mart.) Burret	Henderson s.n. (K)	AJ242183	1*	AJ242146
					2	AJ242147
		Lepidocaryum tenue Mart.	Dransfield JD7012 (K)	AJ242182	2	AJ242139
					3*	AJ242140
OUTGROUPS		*Kerriodoxa elegans* J.Dransf.	1987–2685 (K)	AJ241270	1*	AJ242148
					2	AJ242149
		Nypa fruticans Wurmb.	Baker 512 (SAR)	AJ242185		
		Ceroxylon quindiuense (H. Karst) H. Wendl.	1976–1160 (K)	AJ241284	2*	AJ242150
					3	AJ242151
		Asterogyne martiana (H.Wendl.) H.Wendl. ex Hemsl.	L-81.0284 (BH)	AJ241314	1*	AJ242152
					2	AJ242153

Note. Asterisks indicate which ITS clones were included in the combined dataset.

be considered, and a new classification of the Calamoid-eae will be constructed.

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