

WHAT IS THE RELATIONSHIP AMONG HERNANDIACEAE, LAURACEAE, AND MONIMIACEAE, AND WHY IS THIS QUESTION SO DIFFICULT TO ANSWER?

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Molecular and morphological phylogenetic studies in the Laurales have found that Hernandiaceae, Lauraceae, and Monimiaceae *sensu stricto* form a monophyletic group. Because of the paucity of phylogenetically informative substitutions, however, relationships among families within this clade remain unclear. In general, molecular phylogenies may conflict because of a variety of factors, including substitution rate variation among sites and lineages, taxon sampling, outgroup choice, and base compositional biases. We analyzed a total of 2846 aligned nucleotides from a plastid intron, three spacers, and a portion of the nuclear 26S rRNA gene in a sample of Hernandiaceae, Lauraceae, and Monimiaceae; we used four outgroups with differing substitution rates. Despite obtaining single best topologies with maximum likelihood, minimum evolution, and parsimony approaches, family relationships remained as poorly supported as they were in the previous molecular studies. Exploration of the data indicates that varying substitution rates across lineages or sites, insufficient taxon sampling, fast-evolving outgroups, or biased base composition are unlikely to explain the difficult reconstruction. Exclusion of some of the longest branched taxa (the hemiparasite *Cassytha*, selected Hernandiaceae, and two of the four outgroups) had no effect on topologies. To resolve relationships among the three families one could now complement existing five-gene data sets by adding the basal genera of Lauraceae, Monimiaceae, and Hernandiaceae, which are newly sampled here, or, our preferred strategy, by sequencing low-copy nuclear genes for the key genera to obtain different kinds of data.

Keywords: Hernandiaceae, Lauraceae, *Cassytha*, Monimiaceae, substitution rate variation in 26S, *rpl16*, *trnT-L*, *trnL-F*, *psbA-trnH*.

Introduction

The Laurales comprise seven families, ca. 85 genera, and at least 2400 species (APG 1998; Qiu et al. 1999; Renner 1999). Within Laurales, the oldest split is between the predominantly temperate Calycanthaceae and the six remaining predominantly tropical families (Renner 1999). The latter form two clades, one comprising Siparunaceae, with Atherospermataceae and Gomortegaceae as sister groups, and a second comprising Hernandiaceae, Lauraceae, and Monimiaceae (hereafter the HLM clade). This topology for the order was obtained from an analysis of sequences from the chloroplast (cp) *rbcL* gene, three spacer regions, and an intron, with sampling of 25 genera of Laurales, 11 of which were from the HLM clade (aligned length 4402 base pairs [bp]; Renner 1999). The same two major clades in Laurales were found when five genes from the three plant genomes were combined in a study of the basal angiosperms and of their relationships to gymnosperms (Qiu et al. 1999). The latter study included 15 genera of Laurales, seven of which were from the HLM clade, and genes from the chloroplast (*atpB* and *rbcL*), mitochondrion (*atp1* and *matR*), and nucleus (18S rDNA), with an aligned length of 8733 bp. The two molecular studies differ in their placement of Her-

nandiaceae, a pantropical family of five genera and 60 species. In the cpDNA gene/spacer/intron study, Hernandiaceae were sister to Monimiaceae + Lauraceae. In the three genomes/five genes study, Hernandiaceae appeared as sister to Monimiaceae. Neither relationship was well supported (<80% bootstrap support in the first case, <50% in the second).

Hernandiaceae (4–5 genera/60 species; Kubitzki 1993), Monimiaceae (22–25 genera/200 species; Renner 1998; Renner and Zanis 1999), and Lauraceae (50 genera/2500–3000 species; Rohwer 1993, 2000; Chanderbali et al., in press) are united within Laurales by apically positioned ovules (the single ovule is inserted at or near the locule apex). In contrast, their sister clade—Siparunaceae, Atherospermataceae, and Gomortegaceae—has basal ovules (ovules inserted at or near the base of the locule), except for *Gomortega*, which has highly derived syncarpous two- to three-locular pistils with apical ovules. Based on morphology, Hernandiaceae appear closest to Lauraceae (Doyle et al. 1994, fig. 8b; Renner et al. 1997, fig. 5; Doyle and Endress 2000). Characters supporting a relationship between Lauraceae and Hernandiaceae, to the exclusion of Monimiaceae, are unicarpellate flowers and endospermless mature seeds. Other characters, such as phyllotaxy and anthotaxy, have also been adduced to support a sister group relationship between Lauraceae and Hernandiaceae, but these patterns are not straightforward. Lauraceae have alternate, opposite, or whorled leaves; Hernandiaceae have alternate leaves; and Monimiaceae have decussate or whorled leaves.

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Flowers of Lauraceae have two whorls of usually three tepals each. Hernandiaceae have two whorls of 3-5(-6) tepals or a single whorl of 4-7(-8) tepals. Monimiaceae have 10-20 spirally arranged tepals (*Hortonia* and *Peumus*) or, more commonly, 4-6(-8) tepals in two whorls. Wood anatomy supports a close relationship between Hernandiaceae and Monimiaceae but also between Hernandiaceae and Lauraceae (Shutts 1960). Kubitzki (1969, p. 119), the most recent monographer of Hernandiaceae, thought that Hernandiaceae "might be closest to Lauraceae," but did not provide details. Takhtajan (1973, p. 116; 1997, p. 58), probably following Baillon (1869, esp. pp. 324-325), suggested that Lauraceae originated from primitive Monimiaceae such as *Hortonia*. Even so, he placed Hernandiaceae and Lauraceae in a suborder separate from Monimiaceae (Takhtajan 1997, p. 57). The idea that Hernandiaceae, taken out of Lauraceae and established as a separate family by Blume in 1825, should be reunited with Lauraceae has a long history (Meissner 1864; Baillon 1869; Hallier 1905; Gunderson 1950). However, these morphology-based analyses and systems were hampered by the prevailing wide concept of Monimiaceae, which included Siparunaceae and Atherospermataceae in Monimiaceae. This broad circumscription of Monimiaceae greatly confused character analyses in Laurales, because Monimiaceae *sensu lato* comprise almost the entire range of character states found in Laurales.

As expected from the difficulty inherent in reconstructing exact relationships in the HLM clade and from the clade's great age, each of the families has numerous autapomorphies in terms of morphology as well as DNA sequences. About 40 genera and more than 500 lauraceous species are known from the Early Cretaceous through to the Late Tertiary (Eklund and Kvacek 1998; Eklund 1999). The fossil record of Hernandiaceae and Monimiaceae is poorer, but it still reaches back to the Paleocene, and the HLM sister clade, comprising Siparunaceae, Gomortegaceae, and Atherospermataceae, goes back to the Eocene (Renner et al. 2000).

Low statistical support or contradictory results in molecular studies are commonly attributed to insufficient information from short sequences, poor taxon sampling, strong rate variation across nucleotide sites or taxa, compositional biases, paralogy, hybridization, or a combination of these factors. To improve phylogenetic inference from DNA data, several strategies have been suggested: (1) increasing the number of variable nucleotides will increase the chance of obtaining a known true tree (Cummings et al. 1995; Hillis 1996; Graybeal 1998; Soltis et al. 1998; Bremer et al. 1999; Poe and Swofford 1999). However, it is unclear how many nucleotides will suffice to solve a specific problem; Qiu et al. (1999) analyzed 8733 aligned bp, albeit for a different set of taxa because they focused on the entire basal angiosperms, yet they were unable to resolve family relationship in the HLM clade (seven genera from this clade were included). Empirical and theoretical studies have shown that even data sets with a large number of characters can fail to find known true relationships when substitution rates vary extensively across lineages or sites (e.g., Kuhner and Felsenstein 1994; Takezaki and Gojobori 1999; but see Hillis 1996; Kim 1998; Rannala et al. 1998). Given that the studies performed so far have not resolved HLM relationships, we concentrate here on taxon sampling and methods of tree reconstruction that incorporate heterogeneity in

explicit models rather than length of sequence. Long-branch effects caused by lineages with numerous autapomorphic nucleotide substitutions affect parsimony more than do other tree reconstruction methods, such as maximum likelihood (ML) and corrected-distance methods (e.g., Li 1997, pp. 127-136). The latter consider undetected changes and are therefore less likely to be affected by rate heterogeneity among taxa if the assumed models of sequence evolution are appropriate (Swofford et al. 1996; Li 1997; Poe and Swofford 1999). (2) Dense and judicious sampling to break up long branches also can improve the possibility of finding a known true tree (Lecointre et al. 1993; Hillis 1996; Graybeal 1998; Kim 1998; Rannala et al. 1998). We obtained sequences from four of the five currently recognized genera of Hernandiaceae (Kubitzki [1993] recently raised the single species of *Hernandia* subgenus *Hazomalania* to genus rank, but he now considers this rank change to have been unjustified; K. Kubitzki, personal communication to S. S. Renner, 2000). For Lauraceae, we included eight genera, representing the major lineages of Lauraceae (Rohwer 2000; Chanderbali et al., in press; the latter molecular phylogenetic study includes representatives from 44 of the 50 genera of Lauraceae). For Monimiaceae, we included the four of the family's 15-22 genera that represent the deepest splits in that family (Renner 1998, 1999; Renner and Zanis 1999). Thus, 16 genera were sampled from the HLM clade, as opposed to the seven sampled previously. (3) Long-branch effects can also be introduced by the inclusion of too-distant or fast-evolving outgroup taxa, as observed in studies of basal angiosperms (Chase et al. 1993), vertebrates (e.g., Takezaki and Gojobori 1999), and in statistical analyses of the effects of outgroup rate variation (Lyons-Weiler et al. 1998). We sampled four outgroup taxa, including the sister taxon of the HLM clade, which comprises two slow- and one fast-evolving lineage, and a more distant outgroup in order to explore the effect of outgroup substitution rate heterogeneity on ingroup topology. (4) Because different genomes and genome regions, such as introns, intergenic spacers, and genes, are under different selection regimes, sampling different regions and genomes may even out the effects of location-dependent differences in sequence evolution (Cummings et al. 1995). Our data matrix combines a chloroplast intron and three spacers with a portion of the nuclear 26S rRNA gene. (5) Any method of tree reconstruction may become inconsistent when sequences differ in their base compositions, because standard corrections for multiple changes assume that nucleotides are equally abundant in all sequences. We, therefore, explored a possible compositional bias in the Laurales sequences by performing a χ^2 test on the nucleotide frequencies observed in the different taxa.

We ask two questions: (1) What is the relationship among Hernandiaceae, Lauraceae, and Monimiaceae? and (2) What makes obtaining a robust molecular phylogeny for these taxa so difficult?

Material and Methods

Taxon Sampling

Taxa sequenced for this study are listed in the appendix, which also provides source and voucher information and lists GenBank accession numbers for the 118 sequences analyzed,

56 of which were newly generated for this study. Lauraceae are represented by eight genera and nine species (in three instances, sequences from two species were combined; see below), chosen to represent all major clades (Rohwer 1993, 2000; Chanderbali et al., in press). Basal Lauraceae are represented by the monotypic African *Hypodaphnis*, which is apparently the sister group to all other Lauraceae, and by *Cryptocarya*, *Beilschmiedia*, and *Caryodaphnopsis*. Higher Lauraceae are represented by *Sextonia*, *Ocotea*, and *Litsea*. We also included an Old World and a New World species of *Cassytha*, a pantropical hemiparasitic genus of problematic placement (Rohwer 1993, 2000). Monimiaceae are represented by four genera from their two main lineages (Renner 1998, 1999; Renner and Zanis 1999). For Hernandiaceae, 10 species representing four of their five genera were included, albeit not in all analyses.

We examined the effects of the variable inclusion of the following four outgroup taxa on ingroup topology: *Siparuna* (Siparunaceae), *Doryphora* (Atherospermataceae), *Gomortega* (Gomortegaceae), and *Calycanthus* (Calycanthaceae).

The final data matrix consisted of concatenated sequences from 27 taxa (usually from a single total DNA extract), with the following exceptions (appendix): sequences were combined from *Beilschmiedia brenesii* and *Beilschmiedia tilaranensis*, *Litsea coreana* and *Litsea glaucescens*, *Ocotea grayi* and *Ocotea quixos*, *Siparuna aspera* and *Siparuna guianensis*, *Hernandia guianensis*, *Hernandia moerenhoutiana*, and *Hernandia nymphaeifolia*, and *Sparattanthelium septentrionale* and *Sparattanthelium wonotoboensis*. Species of Hernandiaceae with incomplete sequences were scored as ambiguous for regions not sequenced and were excluded from ML analyses.

Genome Regions Sequenced and Laboratory Methods

Total DNA was isolated from silica gel-dried or herbarium leaves using DNeasy plant mini kits (QIAGEN), according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification followed standard protocols, with 32 cycles of 94°C for 30 s, 52°C for 30–60 s, and 72°C for 60 s. The large intron in the chloroplast *rpl16* gene was amplified using primers 1067F and 18R designed by Asmussen (1999). To amplify the chloroplast *trnT-trnL* and *trnL-trnF* intergenic spacer regions, we used the universal primers a, b, e, and f of Taberlet et al. (1991). The *trnL-trnF* spacer sequences analyzed here begin near the 5' end of the spacer and include 138 bp of the 5' end of the tRNA-Phe (*trnF*) gene. The spacer between the tRNA-Leu (*trnL*) and tRNA-Thr (*trnT*) genes consists of a highly variable 5' end and a conserved 3' end. The latter aligns readily with available *trnT-L* sequences, for example, those from *Dioscorea* (84% sequence similarity in GenBank BLAST searches) and *Allium* (72% sequence similarity). By contrast, the 5' end varies drastically among taxa and contains numerous short repeats and apparent inversions. For the present analyses, we included only the conserved 3' end of the *trnT-trnL* region. The *psbA-trnH* intergenic spacer was amplified using primers designed by Sang et al. (1997), which generated sequences consisting of 44 bp of the 3' end of the *psbA* gene, ~530 bp of the rapidly diverging *psbA-trnH* spacer, and 29 bp of the 5' end of the tRNA-His (*trnH*) gene. Because of alignment difficulties among Hernandiaceae and the re-

maining taxa, we used only the first half of the spacer in the analyses. The 5' region that includes the first two expansion domains of the nuclear 26S rRNA gene was amplified using the forward primer 27F (D. Nickrent, unpublished manuscript) and the reverse primer 641R (Kuzoff et al. 1998). In the analyses, we included the conserved core between positions 57 and 111, the first expansion segment between positions 111 and 270, and the second expansion segment between positions 430 and 641. Four base pairs between positions 474 and 477 were excluded from the analyses because of alignment ambiguity.

PCR products were purified either by running the entire product on a low-melting-point agarose gel and then recovering the amplified DNA with QIAquick gel extraction kits (QIAGEN) or by using the QIAquick PCR purification columns directly without a prior gel purification step. Cycle sequencing of the amplified products was conducted with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Norwalk, Conn.), using 2.5 ng of primer in a 5- μ L reaction volume. Sequencing reactions were purified by ethanol precipitation and were run on ABI 373 or ABI 377 automated sequencers. Both strands of DNA were sequenced and used to generate a consensus sequence using Sequencher software (GeneCodes, Ann Arbor, Mich.). Sequences were aligned manually, and except for the ends of the *trnT-trnL* and *psbA-trnH* spacers (which were excluded; see above) and a region of four nucleotides in the 26S gene, there were no "difficult-to-align" regions.

Phylogenetic Analyses

Phylogenetic analyses of the aligned sequences were conducted with test version 4.0b.4 of PAUP* (Swofford 1998). ML analyses were performed using the general time-reversible model (GTR; Yang 1994), which estimates probabilities for the six possible nucleotide substitutions in addition to accounting for unequal base frequencies, transition/transversion bias, and substitution rate heterogeneity across nucleotide sites. To assess whether this parameter-rich model fit our data significantly better than the simpler Hasegawa-Kishino-Yano model (HKY85; Hasegawa et al. 1985), we performed a likelihood ratio test on the highest likelihoods obtained under these two models, using four degrees of freedom (cf. Sullivan et al. 1999). For both models, the proportion of invariable sites, P_{inv} , and the shape of the parameter, α , were estimated simultaneously using the discrete gamma approximation of Yang (1994; implemented in PAUP*), with four rate categories to approximate the continuous gamma distribution. Both models yielded the same single best tree, and the likelihood ratio test rejected the HKY85 model in favor of the GTR model [$\ln L = 2(13,408.65758 - 13,372.37166) = \chi^2 = 72.57$; $P < 0.001$; $df = 4$]. We therefore selected the GTR-invariable-sites-plus-gamma model as that which was most appropriate to our data.

Rate heterogeneity across sites affects the performance of different tree reconstruction methods, and its estimation has received considerable attention (Yang 1996; Sullivan et al. 1999; Takezaki and Gojobori 1999). To mitigate the potentially deleterious effects of rate variation, one can combine an invariable-sites model with a gamma-distributed-rates model. In the former, some proportion of sites (P_{inv}) is assumed to be

Table 1

Descriptive Statistics for Separate and Combined Data Partitions for the Full Set of 27 Taxa of Laurales

Data partition	<i>rpl16</i> intron	<i>trnT-trnL</i> spacer	<i>trnL-trnF</i> spacer	<i>psbA-trnH</i> spacer	26S rDNA gene	Combined data
Aligned nucleotides	1023	519	529	170	605/601*	2846/2842*
Autapomorphic variable sites (percentage of total characters)	134 (13)	80 (15)	90 (17)	26 (15)	66 (11)	396 (14)
Informative sites (percentage of total characters)	205 (20)	148 (30)	129 (25)	47 (28)	126 (21)	652 (23)
A	27	37*	30	27	20*	28
C	21	14*	21	16	28*	21
G	16*	24	16	21	37*	23
T	36*	25	33	36	15*	28
Number of gaps (gap size range in bp)	40 (1-48)	19 (1-180) ^b	26 (1-120) ^b	6 (1-12)	9 (1-6)	100 (1-180)
P_{inv}^c	0.25	0.12	0.12	0.00	0.44	0.22
α^c	1.18	2.17	3.45	0.64	0.55	0.94

Note. Nucleotide frequencies marked with an asterisk deviate significantly from the mean (Pearson χ^2 statistic, $P < 0.001$).

* Base pairs (bp) 474–477 were eliminated in the final matrix because of alignment ambiguities; only 601 nucleotides of the 26S rDNA region were used.

^b *Cassytha* has a 180-bp deletion in the aligned *trnT-trnL* spacer region, and *Gyrocarpus* has a 135-bp deletion in the aligned *trnL-trnF* region.

^c Maximum likelihood estimates of proportions of invariable sites and substitution rate heterogeneity parameters (α) were obtained under the general-time-reversible model. An α of >1 indicates that most sites have intermediate rates, whereas few sites have very low or very high rates. An α of ≤ 1 indicates that most sites have very low rates or are almost invariable, whereas others change at very high rates (Yang and Kumar 1996). The estimate of $\alpha = 0.00$ (i.e., infinity) for the *psbA-trnH* spacer, which would imply no rate heterogeneity, probably has a very large variance because it is based on only 170 bp.

completely resistant to change; in the latter, the distribution of relative rates over variable sites is assumed to follow a gamma distribution whose shape parameter (α) determines rate heterogeneity. The dependence of P_{inv} and α on tree topology is minor as long as strongly supported groups are maintained (Yang and Kumar 1996; Sullivan et al. 1999). Therefore, both parameters can initially be estimated for distance trees from the same data, which greatly reduces the computational demands of ML searches with full branch swapping.

Starting trees for ML searches were minimum evolution trees, using log-determinant (LogDet) distances (Lake 1994; Lockhart et al. 1994), and the swapping strategy employed was TBR swapping. We used quartet puzzling (Strimmer and von Haeseler 1996; implemented in PAUP*), a fast tree search algorithm that allows for analysis of large data sets, to obtain estimations of support for internal branches in the ML trees. These values have the same practical meaning as bootstrap values; quartets showing a quartet puzzling reliability of 90%–100% are strongly supported (Strimmer and von Haeseler 1996).

Parsimony analyses were performed using heuristic searches, with 10 random addition replicates and TBR swapping. Characters were unweighted and unordered, and gaps were treated as missing data. Nonparametric bootstrap support (Felsenstein 1985) for each clade was estimated based on 1000 replicates, using closest taxon addition, TBR swapping, and MULPARS. The COLLAPSE (but not the STEEPEST DESCENT) options of PAUP were in effect during all searches. Most-parsimonious trees were generated independently for the five data sets, fol-

lowed by bootstrap analyses, to assess whether conflict among data sets was strongly supported (i.e., had $>80\%$ bootstrap support). In the absence of such conflict, the data were combined in a global analysis. Characters supporting particular topologies were examined using MacClade 3.04 (Maddison and Maddison 1992).

Results

Data Characteristics and Phylogenetic Analyses

Each of the five sequenced DNA regions is characterized in table 1. The total aligned sequence length comprised 2846 nucleotides, of which four (positions 474–477 in the 26S gene) were eliminated from the analysis because of alignment ambiguity. The final matrix for all 27 taxa contained 652 (23%) parsimony-informative sites and 396 (14%) variable but uninformative sites. As expected, nucleotide composition of the 26S gene segment sequenced differed significantly from that of the cpDNA spacers and intron (table 1). Of the 605-bp sequence, 203 bp belong to highly conserved parts of the gene, and the remainder belong to two expansion segments. The average G + C content across the sequenced part of the gene was 64% (i.e., slightly higher than the 56% characterizing the entire gene and slightly lower than the 66% found across all expansion segments; Kuzoff et al. 1998). A comparison of nucleotide frequencies shows that there are no significant differences among taxa (table 2). Under parsimony, the ratio of transitions to transversions (ti/tv) across all sequences was

1.3 : 1 (1040–1091/799–850). The ti : tv ratio was 1.5 : 1 (268–287/177–196) for the 26S gene alone. Because parsimony tends to underestimate ti : tv ratios, we also estimated the ti : tv ratio under ML using the HKY model. This gave a ti : tv ratio of 1.5 : 1 across all sequences.

Of the 100 sequence-length mutations (indels), most occurred in the *rpl16* intron, and none were phylogenetically informative at the family level. The longest indels occurred in the *trnT-trnL* spacer, which comprised between 444 bp in *Cassytha filiformis* and 840 bp in *Siparuna aspera*.

Rate heterogeneity across sites in each of the five genome regions is measured by α , which is inversely related to the extent of rate variation across sites. Table 1 shows the values for α that were estimated under the GTR model. Only the 26S gene shows moderately strong among-site rate heterogeneity. The estimate of $\alpha = 0.00$ (i.e., infinity) for the *psbA-trnH* spacer, which would imply no rate heterogeneity, probably has a very large variance since it is based on only 170 bp. For the concatenated data, α is close to 1, indicating an apparently random distribution of the rates at which sites are changing.

The single best tree resulting from the ML analysis under the GTR-invariable-sites-plus-gamma model (fig. 1) shows Lauraceae to be sister to Hernandiaceae. However, the branch supporting the two families is extremely short, and the grouping was the only one not recovered via quartet puzzling, which we used to obtain confidence values for the ML topology (using the same GTR settings). The puzzle tree instead showed Hernandiaceae to be sister to Monimiaceae, but the support for this clade was only 43%.

After excluding all gapped and ambiguous sites and excluding the seven Hernandiaceae with incomplete sequences, we performed a likelihood ratio test to corroborate the visual impression that substitution accumulation across taxa had not been clocklike. The clock and nonclock analyses had significantly different likelihoods ($\chi^2 = 242.5$, $df = 20$, $P <$

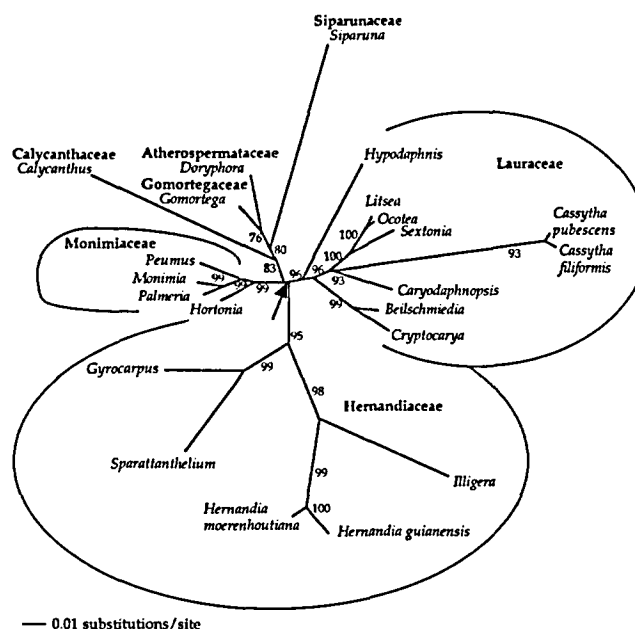


Fig. 1 The highest likelihood tree shown as an unrooted phylogram for Hernandiaceae, Lauraceae, and Monimiaceae. The model used was the GTR-invariable-sites-plus-gamma model. Support values (at nodes) were obtained via quartet puzzling as implemented in PAUP. The Hernandiaceae + Lauraceae clade is supported by an extremely short stem (arrow).

0.0001). From the phylograms (figs. 1–3) it appears that *Calycanthus*, *Siparuna*, and *Cassytha* likely caused most of the rate heterogeneity in the data. The exclusion of these three taxa from the analysis, however, did not change the ML topology or significantly affect the puzzle support values.

Table 2
Nucleotide Frequencies in Different Lineages of Laurales

	A	C	G	T	Number of sites
<i>Beilschmiedia</i>	0.28	0.21	0.22	0.29	2309
<i>Calycanthus</i>	0.27	0.21	0.23	0.29	2308
<i>Caryodaphnopsis</i>	0.28	0.22	0.23	0.28	2342
<i>Cassytha filiformis</i>	0.28	0.21	0.22	0.28	2177
<i>Cryptocarya</i>	0.28	0.21	0.22	0.29	2277
<i>Doryphora</i>	0.28	0.21	0.22	0.28	2329
<i>Gomortega</i>	0.28	0.21	0.22	0.29	2350
<i>Gyrocarpus</i>	0.28	0.21	0.23	0.28	2277
<i>Hernandia moerenhoutiana</i>	0.27	0.21	0.23	0.29	2339
<i>Hortonia</i>	0.28	0.21	0.23	0.28	2342
<i>Hypodaphnis</i>	0.28	0.21	0.22	0.29	2386
<i>Illigera</i>	0.27	0.21	0.23	0.29	2329
<i>Litsea</i>	0.28	0.21	0.23	0.28	2355
<i>Monimia</i>	0.28	0.21	0.22	0.29	2335
<i>Ocotea</i>	0.28	0.21	0.23	0.28	2374
<i>Palmeria</i>	0.28	0.21	0.22	0.29	2344
<i>Peumus</i>	0.28	0.21	0.22	0.29	2352
<i>Sextonia</i>	0.28	0.21	0.22	0.28	2355
<i>Siparuna</i>	0.28	0.21	0.22	0.29	2355
<i>Sparattanthelium</i>	0.27	0.21	0.23	0.29	2297

The minimum evolution tree (fig. 2), using LogDet distances and $P_{inv} = 0.22$ as estimated under ML, shows Hernandiaceae to be sister to Monimiaceae, regardless of whether the longest branched groups, *Calycanthus*, *Siparuna*, and *Cassytha*, are included or excluded. Support for this family grouping remained consistently low (56% included all taxa).

Parsimony analysis of the concatenated sequences resulted in a single shortest tree found in all random addition replicates (length = 1969 steps; consistency index = 0.70; retention index = 0.74; fig. 3). Parsimony showed Monimiaceae and Lauraceae to be sister groups (59% bootstrap support). The same topology and similar low bootstrap values were obtained when species of Hernandiaceae with incomplete sequences were excluded.

A Kishino-Hasegawa test (implemented in PAUP), which tests the difference between two log-likelihood scores, shows that the likelihoods of the alternative topologies, (Lauraceae + Monimiaceae) Hernandiaceae, (Lauraceae + Hernandiaceae) Monimiaceae, and (Monimiaceae + Hernandiaceae) Lauraceae, are not significantly different. Shi-

modaira and Hasegawa (1999) recently pointed out that the Kishino-Hasegawa test tends to reject alternative topologies too readily, leading to overconfidence in wrong "best" topologies. By the same token, the failure of the test to reject any of the three topologies supports the notion that current data cannot distinguish among them.

To learn more about the sites that support each of the three possible topologies, we examined the character changes supporting them. Twenty-one base pairs change along the L + M stem in the parsimony tree (fig. 3); all but one of them are homoplasious. The unambiguous character is a transversion (T to A) in the *psbA-trnH* spacer (reversed only in higher Lauraceae). The other characters supporting L + M are five substitutions in the *rpl16* intron, four in the *trnT-trnL* spacer, five in the *trnL-trnF* spacer, and six changes in the 26S expansion regions. An M + H grouping is supported by three changes in *trnL-trnF* and by six changes in the 26S expansion regions; an L + H grouping is supported solely by six changes in the 26S expansion regions. All of these potentially syna-

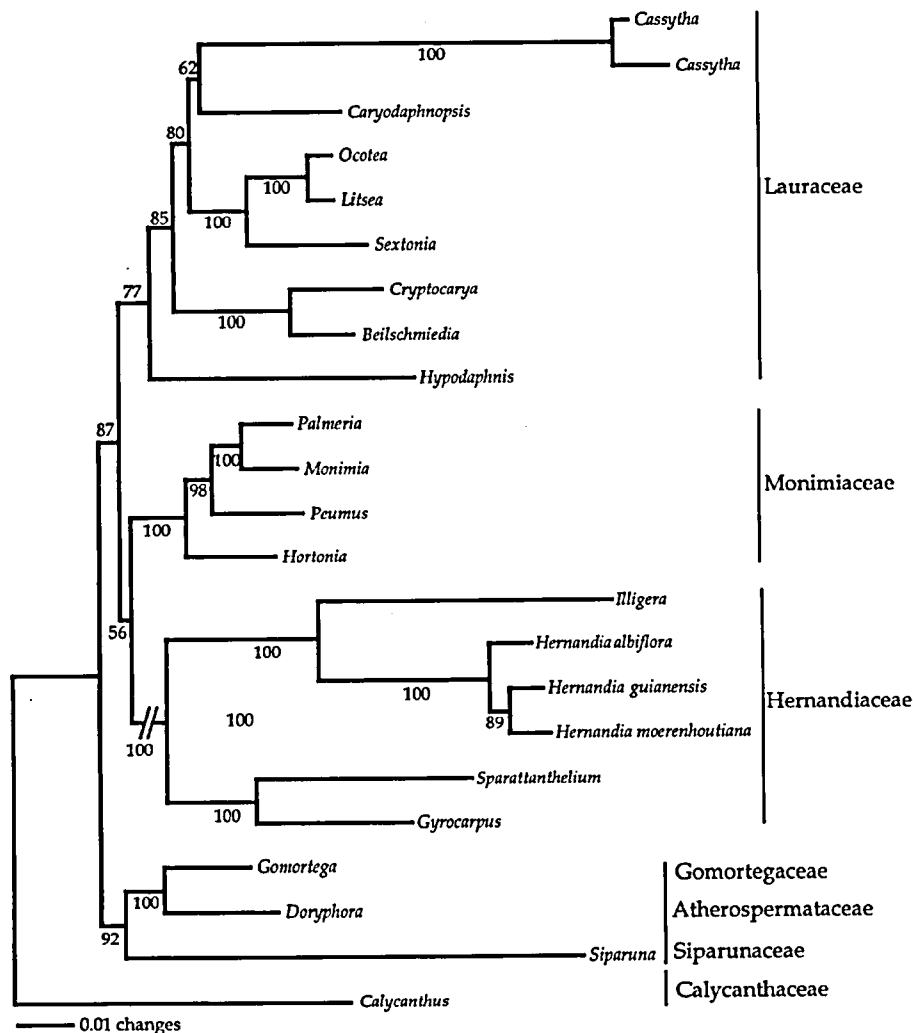


Fig. 2 Minimum evolution tree, using LogDet distances for Hernandiaceae, Lauraceae, and Monimiaceae. Bootstrap values below branches are based on 1000 replicates. The branch leading to Hernandiaceae is shortened by one-half.

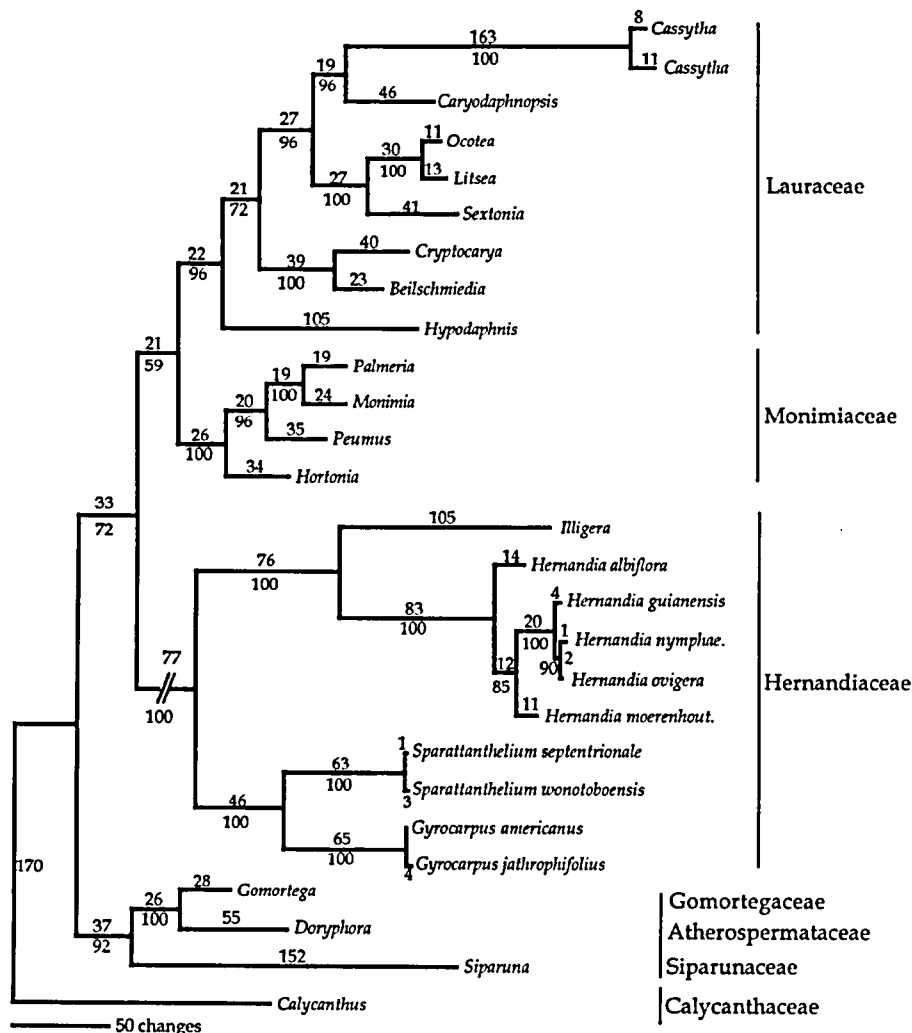


Fig. 3 The single most parsimonious tree for Hernandiaceae, Lauraceae, and Monimiaceae (length = 1969 steps, consistency index = 0.70, retention index = 0.74). Figures above branches give the number of substitutions along a branch; figures below branches are bootstrap values based on 1000 replicates. The branch leading to Hernandiaceae is shortened by one-half.

pomorphic changes involve nucleotides that also change elsewhere in the data set.

Discussion

The results of this study confirm earlier morphological and molecular work showing that Hernandiaceae, Lauraceae, and Monimiaceae are closely related. Even large amounts of sequence data, however, do not robustly resolve relationships among these three families. Thus, neither concatenated sequences from five genes from all three plant genomes (Qiu et al. 1999; albeit, this holds true for a smaller sample of HLM taxa, since Laurales were not the focus of the Qiu et al. study) nor the more variable DNA regions concatenated here contain the right number of conserved substitutions. Poor ingroup taxon sampling is unlikely as a cause of the problem, because each of the three families is sufficiently well understood, from a molecular sampling point of view, to permit correct taxon

choice. Lauraceae and Monimiaceae are each represented by several genera that represent the deepest splits in these families (Renner 1998, 1999; Renner and Zanis 1999; Rohwer 2000; Chanderbali et al., in press). Hernandiaceae are represented by several species, representing four of their five genera. Kubitzki recently (1993) segregated a monotypic Madagascan subgenus of *Hernandia* as a separate genus, but he now sees *Hazomalania voyroni* as being derived within *Hernandia* (K. Kubitzki, personal communication to S. S. Renner, 2000). Sampling problems may still exist, however, as a result of the extinction of basal HLM genera during the 100-million-year-long history of the clade.

Simulation studies have shown that correct phylogenetic reconstruction can be hampered by heterogeneity in molecular evolutionary rates among lineages or sites, but that except in extreme cases, it should be possible to reconstruct the correct tree by appropriate selection of methods, models, and parameters (Felsenstein 1988; Li 1997; Siddall 1998). However,

among-lineage rate heterogeneity is unlikely to be the cause of the difficult reconstruction of HLM relationships. First, exclusion of fast-evolving taxa had no effect on topology or bootstrap support for family relationships. Second, our trees show long and short branches that are interspersed, regardless of tree reconstruction method, except in the case of Monimiaceae, which form a uniformly short-branched clade. Simulations using matrices with up to ninefold substitution rate differences among taxa indicate that ML may be robust against unequal rate effects (Li 1997, p. 135). Neighbor joining, too, is considered robust against unequal rates, as long as distances are estimated accurately (Felsenstein 1988; Li 1997). Rate differences across sites rather than taxa also are unlikely to be the cause of the difficult reconstruction of HLM relationships. The gamma parameter estimated for the concatenated data, $\alpha = 0.94$, indicates that substitution rate is almost homogeneous. Only the 26S gene ($\alpha = 0.55$) shows moderately strong among-site rate heterogeneity. In addition, different base composition among lineages is not a problem (table 2).

There is also no statistically supported conflict among trees obtained from the nuclear data (i.e., the large subunit 26S rDNA) and trees obtained from the chloroplast data. However, the tree obtained from the 26S data is the only one in which the three families are not monophyletic, which indicates that there may be a difference between the nuclear and chloroplast data that might be explored further. We know of no examples of hybridization within or among Lauraceae, Hernandiaceae, or Monimiaceae, but these are predominantly tropical, woody groups, the biology of which is not well known. Perhaps tellingly, Hernandiaceae and Monimiaceae share the presence of extensive polyploid series, such as $n=10, 20, 30$ or $n=24, 48$ in the former and $n=19, 39, 40-42, 50$, and up to 90 in the latter (Morawetz 1986). By contrast, Lauraceae almost uniformly have a chromosome base number of $n=12$ (Rohwer 1993).

Lack of a clear answer to the question regarding relationships among Hernandiaceae, Lauraceae, and Monimiaceae in this and previous studies (Qiu et al. 1999; Renner 1999) thus appears to be the result primarily of insufficient phylogenetic information along the internal branch that separates two of these families from the third. This implies that there exists a striking difference between molecular and morphological evolution in this part of the Laurales. In an angiosperm-wide morphological cladistic analysis, Doyle and Endress (2000) have found 100% bootstrap support for a sister group relationship between Lauraceae and Hernandiaceae, making this one of the best-supported relationships in their tree. Why would such a strong morphological signal not be paralleled by the molecular signal analyzed here? In other lauralean clades, the amount of molecular change seen in the very same genome regions seems to parallel morphological change closely. For example, Calycanthaceae differ by many autapomorphic morphological changes from the remaining families of Laurales, and they also have a long branch in molecular studies (Qiu et al. 1999; Renner 1999; this study). *Cassytha*, the hemiparasitic twiner whose precise placement in Lauraceae has been unclear because of numerous autapomorphies (Rohwer 1993, 2000; Chanderbali et al., in press), has accumulated numerous unique molecular changes (figs. 1–3).

Although morphological investigations by themselves

will not solve the current impasse, it is important to consider the possible morphological support for alternative topologies. The traditional view is that Lauraceae and Hernandiaceae are closer to each other than either is to Monimiaceae. Morphological arguments for this theory come mainly from the unicarpellate condition fixed in these two families alone among Laurales as well as from their loss of a persistent endosperm. Among other Laurales, an endosperm is also absent in mature seeds of Calycanthaceae, and solitary carpels are also found in Calycanthaceae (*Idiospermum*) and in higher Monimiaceae (*Xymalos*, *Hennecartia*). The remaining Laurales, including all basal Monimiaceae, have numerous carpels per flower. Importantly, Hernandiaceae have epigynous flowers (elsewhere in Laurales, these flowers are only found in *Gomortega*), as does a monotypic, possibly basal genus of Lauraceae, *Hypodaphnis* (Rohwer 2000; Chanderbali et al., in press). Other Lauraceae and most Monimiaceae are perigynous. In terms of stamen structure, Lauraceae (with tetrasporangiate anthers) and Hernandiaceae (with disporangiate anthers) share valvate anther opening, whereas Monimiaceae anthers dehisce longitudinally or transversally. In only a few Monimiaceae do anthers have short lateral incisions at the top and bottom of the longitudinal slits, resulting in a saloon door-like opening of the thecae (Baillon 1869, fig. 339; Endress and Hufford 1989, figs. 83–84). As in an earlier morphological cladistic analysis of Laurales (Renner et al. 1997) and as found by Doyle and Endress (2000) in a much broader analysis of morphological and anatomical characters of basal angiosperms, a sister group relationship between Monimiaceae and Hernandiaceae or between Lauraceae and Monimiaceae appears to receive no support from morphology or anatomy.

The fundamental question raised by this and prior studies is how the contrast between a series of potentially shared complex morphological characters and the absence of shared molecular characters (in the sampled data) can best be understood. Perhaps genome regions other than the ones sequenced so far changed coincidentally with morphological characters and are under similar stabilizing selection. Sequencing such regions might enable one to pick up congruent signals from molecules and morphology. Thus, we see two ways to further attack “the HLM problem.” One way is to augment DNA data by supplementing the existing *atpB*, *rbcL*, *atp1*, *matR*, and 18S rDNA data set of Qiu et al. (1999) for a sample of the key genera newly sampled here and to also sequence the remaining 80% of the 26S gene for all key taxa. These taxa include *Hypodaphnis*, *Beilschmiedia*, *Cassytha*, *Caryodaphnopsis*, and *Sextonia* (Lauraceae) and *Illigera*, *Sparattanthelium*, and *Hernandia* (Hernandiaceae). Adding these data to the spacers and introns sequenced here would bring the total number of nucleotides up to almost 12,000 bp and may well yield enough phylogenetic signal to resolve the HLM trichotomy. Alternatively—and this is the strategy we favor—one could sequence one or more low-copy nuclear genes, which are providing strong phylogenetic signals in other basal angiosperms, especially monocots (Soltis and Soltis 1998; Mathews et al. 2000 and references cited therein). These genes may be evolving in different ways from the regions sampled so far and thus may represent more informative data.

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Appendix

Table A1

Sources of Plant Material and GenBank Accession Numbers for the Phylogenetic Analysis of Laurales

Species	Chloroplast regions				Nuclear region	Source or voucher
	<i>rpl16</i>	<i>trnT-trnL</i>	<i>trnL-trnF</i>	<i>psbA-trnH</i>	26S	
Hernandiaceae:						
<i>Gyrocarpus americanus</i> Jacq.	AF127261	AF129025	AF012398	AF129054	AF262001	Chase 317 (NCU)
<i>Gyrocarpus jatrophifolius</i> Domin	...	AF233597	AF232026	...	...	Zamora and Hammel 1883 (INB)
<i>Hernandia albiflora</i> (C. T. White) Kubitzki	...	AF233598	AF232027	...	...	Jago 4689 (NSW)
<i>Hernandia guianensis</i> Aublet	AF127263	AF233599	AF232028	AF261993	...*	Munich BG
<i>Hernandia moerenhoutiana</i> Guillem.	AF130310	AF129026	AF052198	AF129055	...*	Brisbane BG
<i>Hernandia nymphaeifolia</i> (Presl) Kubitzki	...	AF233600	AF232029	...	AF264139**	Natl. BG Hawaii 960860; Zurich BG
<i>Hernandia ovigera</i> L.	...	AF233601	AF232030	AF261994	...	Munich BG
<i>Illigera luzonensis</i> (Presl) Merr.	AF127264	AF129030	AF052199	AF129057	AF262002	Munich BG
<i>Sparattanthelium septentrionale</i> Sandw.	...	AF233602	AF232031	...	AF262003*	Aguilar 5589 (INB)
<i>Sparattanthelium wonotoboensis</i> Kosterm.	AF127262	AF129043	AF053342	AF129070	...*	Munich BG 97/134
Lauraceae:						
<i>Beilschmiedia brenesii</i> C. K. Allen	...	...	...	...	AF262004*	Yasuda 1314 (MO)
<i>Beilschmiedia tilaranensis</i> Sa. Nishida	AF127265	AF129015	AF129014	AF129045	...*	Yasuda 1313 (MO)
<i>Caryodaphnopsis bilocellata</i> van der Werff	AF232743	AF233603	AF232032	AF261995	AF262005	van der Werff 14195 (MO)
<i>Cassytha filiformis</i> L.	AF232744	AF233605	AF232034	AF261996	AF262006	Chanderbali 205 (MO)
<i>Cassytha pubescens</i> R. Br.	...	AF233604	AF232033	...	AF262007	Foreman 1913 (MEL)
<i>Cryptocarya thouvenotii</i> (Danguy) Kosterm.	AF232745	AF233606	AF232035	AF261997	AF262008	van der Werff 12723 (MO)
<i>Hypodaphnis zenkeri</i> (Engl.) Stapf	AF232746	AF233607	AF232036	AF261998	AF262009	McPherson 16184 (MO)
<i>Litsea coreana</i> Leveille	...	...	...	...	AF262010*	Yasuda 1356 (MO)
<i>Litsea glaucescens</i> Kunth	AF127266	AF129036	AF129035	AF129063	...*	Lorea 5496 (MO)
<i>Ocotea grayi</i> van der Werff	AF232747*	...	...	...	...	van der Werff 12732 (MO)
<i>Ocotea quixos</i> (Lam.) Kosterm.	...*	AF233608	AF232037	AF261999	AF262011	Missouri BG 990585
<i>Sextonia pubescens</i> van der Werff	AF232748	AF233609	AF232038	AF262000	AF262012	Vasquez 25229 (MO)

Table A1

(Continued)

Species	Chloroplast regions				Nuclear region	Source or voucher
	<i>rpl16</i>	<i>trnT-trnL</i>	<i>trnL-trnF</i>	<i>psbA-trnH</i>	26S	
Monimiaceae:						
<i>Hortonia floribunda</i> Wight ex Arnold	AF129027	AF129028	AF040683	AF129071	AF264143*	Colombo BG
<i>Monimia ovalifolia</i> Thouars	AF127269	AF129038	AF054896	AF129065	AF264144*	Strasberg s.n. (REU)
<i>Palmeria scandens</i> F. Muell.	AF127270	AF129040	AF052200	AF129067	AF264142*	Bradford 878 (MO)
<i>Peumus boldus</i> Molina	AF127454	AF129041	AF012403	AF129068	AF264141*	Edinburgh BG 19870707
Outgroups:						
Atherospermataceae:						
<i>Doryphora sassafras</i> Endl.	AF127252	AF129023	AF040672	AF129050	AF262013	Sydney BG 18026
Calycanthaceae:						
<i>Calycanthus occidentalis</i> Hook. & Arn.	AF127250	AF129017	AF012396	AF129046	AF264145*	Missouri BG 897432
Gomortegaceae:						
<i>Gomortega nitida</i> R. & P.	AF127260	AF264020	AF012404	AF129053	AF262014	Rodriguez 3070 (CONC)
Siparunaceae:						
<i>Siparuna aspera</i> (R. & P.) A. DC.	...*	AF129042	AF040695	AF129069	AF262015	Madriñán et al. 1502 (COL)
<i>Siparuna guianensis</i> Aublet	AF127455*	...	...	...	...	Chanderbali 247 (MO)

Note. Herbarium vouchers are deposited in the following herbaria: the Missouri Botanical Garden (MO), Colombian National Herbarium (COL), University of North Carolina (NCU), Réunion (REU), Concepción (CONC), New South Wales (NSW), Melbourne (MEL), Queensland (QRS), and the National Biological Institute in Costa Rica (INB). Botanical Gardens (BG) are cited for cultivated plants. Sequences marked with an asterisk were used to complement the set of sequences in the line directly above or below them (also with an asterisk).

* D. Soltis and M. Zanis, unpublished manuscript.

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