Evolution, 56(10), 2002, pp. 1931–1942

EARLY TERTIARY OUT-OF-INDIA DISPERSAL OF CRYPTERONIACEAE: EVIDENCE FROM PHYLOGENY AND MOLECULAR DATING

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Abstract.—Phylogenetic analyses and molecular dating estimates based on chloroplast DNA sequences were used to establish the relationships of the southern and Southeast Asian Crypteroniaceae and elucidate their biogeographic history. Maximum parsimony and likelihood analyses of *rbc*L sequences suggested that Crypteroniaceae should be restricted to *Crypteronia*, *Axinandra*, and *Dactylocladus* and that Crypteroniaceae, so defined, are sister to a clade formed by three small African taxa (Oliniaceae, Penaeaceae, and Rhynchocalycaceae) and the monotypic Central and South American Alzateaceae. Three molecular dating approaches (maximum-likelihood under a molecular clock, Langley-Fitch, and penalized-likelihood) were used to infer the age of Crypteroniaceae using both paleobotanic and geologic calibrations. Comparisons among these three methods revealed significant lineage effects in *rbc*L sequences. Clock-independent dating estimates suggested that divergence of Crypteroniaceae from its African and South American relatives coincided with the breakup of Gondwana, and that India likely served as a ''raft'' transporting Crypteroniaceae to Asia, with later expansion to Southeast Asia. To our knowledge, Crypteroniaceae are the first plant group for which the out-of-India hypothesis is well corroborated by molecular-based estimates of divergence times.

Key words.—Biogeography, Gondwana, lineage effects, maximum likelihood, molecular clock, penalized likelihood, vicariance.

Received March 11, 2002. Accepted June 26, 2002.

The out-of-India hypothesis holds that some Asian biotic elements have an ancient Gondwanan origin and arrived in Asia by rafting on the Indian plate (McKenna 1973). After splitting from eastern Gondwana in the Early Cretaceous, India started its northbound journey and drifted close to the African plate, all the while receiving diverse Late Cretaceous elements of the African tropical flora and losing temperate elements of the eastern Gondwanan gymnosperm flora (Morley 2000). The dramatic latitudinal and climatic changes that affected India during the Late Cretaceous and Tertiary, as the plate traveled from Gondwana and then collided with Asia, caused massive extinctions in its biota, making it more difficult to find evidence for out-of-India dispersal (Raven and Axelrod 1974). Fossils provide direct support for dispersal from India into Asia in only a few angiosperms (Bande and Prakash 1986; Bande 1992) and vertebrates (Krause and Maas 1990). In other taxa, current distribution patterns may suggest an out-of-India origin, but direct fossil documentation is not available. In these cases, phylogeny reconstruction and molecular dating estimates can be used to provide crucial evidence for or against this biogeographic hypothesis. Phylogenetic and dating analyses have confirmed an out-of-India migration in ranid frogs (Bossuyt and Milinkovitch 2001), acrodont lizards (Macey et al. 2000), and ratite birds (Cooper et al. 2001), but have so far failed to support the same pattern in plants. For example, a published molecular phylogenetic study of *Durio* (Bombacaceae) was inconclusive regarding the possibility that the Southeast Asian species of this genus migrated from India after its collision with Asia (Nyffeler and Baum 2001). Current distribution, fossil record, and phylogenetic relationships support the out-of-India hypothesis for Dipterocarpaceae (Ashton and Gunatilleke 1987; Dayanandan et al. 1999), although no molecular estimates for the age of the family have been attempted. The Crypteroniaceae (Myrtales) might provide an example of out-of-India origin for a group of southern and Southeast Asian plants, because morphological analyses supported their close affinity to four western Gondwanan taxa (Penaeaceae, Oliniaceae, Rhynchocalycaceae, and Alzateaceae; Johnson and Briggs 1984), suggesting that the current distribution of these five taxa, possibly aided by short- to medium-distance dispersal, could be the result of Gondwana fragmentation (Tobe and Raven 1984; Ashton and Gunatilleke 1987).

The Crypteroniaceae A. DC. are thought to belong to Myrtales on the basis of two wood anatomical features: bicollateral vascular bundles in the primary stem and vestured pits in the vessel elements of the secondary xylem (Dahlgren and Thorne 1984; Johnson and Briggs 1984). However, the circumscription and taxonomic affinities of Crypteroniaceae have been controversial, as testified by the numerous changes of familial boundaries that have occurred since de Candolle (1868) first described the family (see Table 1). Five genera of evergreen trees are at the center of this taxonomic controversy: *Axinandra* Thw., *Crypteronia* Bl., *Dactylocladus* Oliv., *Rhynchocalyx* Oliv., and *Alzatea* Ruiz and Pav. (Oliver 1895; van Beusekom-Osinga and van Beusekom 1975; van Beusekom-Osinga 1977; Graham 1984; Johnson and Briggs 1984; Pereira and Wong 1995; Zhu 1997; see Table 2). A comprehensive morphological phylogenetic analysis of the Myrtales supported a close affinity of Crypteroniaceae sensu stricto, including only *Axinandra*, *Crypteronia*, and *Dactylocladus*, with Alzateaceae, Rhynchocalycaceae, Penaeaceae, Oliniaceae, Melastomataceae, and Memecylaceae, although

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TABLE 1. Taxonomic affiliations of the five genera variously ascribed to Crypteroniaceae by different authors in chronological order.

Genera	Family	Authors
Axinandra *Crypteronia* *Dactylocladus* *Rhynchocalyx* *Alzatea*	Crypteroniaceae sensu lato	van Beusekom-Osinga and van Beusekom 1975 Muller 1975 van Beusekom-Osinga 1977
Axinandra *Crypteronia* *Dactylocladus*	Crypteroniaceae sensu stricto	van Vliet and Baas 1975 Dahlgren and Thorne 1984 Graham 1984 Johnson and Briggs 1984 Tobe and Raven 1987b Mentink and Baas 1992 Pereira and Wong 1995
Axinandra *Crypteronia* *Dactylocladus*	Melastomataceae	Triana 1865 Baillon 1877 Cogniaux 1891 Krasser 1893 Gilg 1897 Bakhuizen van den Brink 1947 Meijer 1972 van Vliet et al. 1981 van Vliet and Baas 1984
Rhynchocalyx *Alzatea*	Lythraceae	Bentham and Hooker 1867 Hallier 1911 Lourteig 1965 van Vliet and Baas 1984

relationships within this clade were not resolved (Johnson and Briggs 1984). Subsequent molecular studies greatly clarified relationships within Myrtales (Conti et al. 1996, 1997), but these studies did not include representatives of Crypteroniaceae. Here we present the results of phylogenetic and molecular dating analyses of DNA sequence data from the chloroplast gene *rbc*L for Crypteroniaceae and all other families of Myrtales.

To corroborate or refute the out-of-India hypothesis for Crypteroniaceae, independent estimates for the time of origin of the family are needed. The Gondwanan origin of Crypteroniaceae, followed by survival on the rafting Indian plate and dispersal from India into Asia, could be accepted if both phylogenetic relationships and molecular estimates of their age were concordant with the order in which the Gondwanan continents fragmented (e.g., Vinnersten and Bremer 2001), or rejected if results were not compatible with geologic events (e.g., Baum et al. 1998; Givnish et al. 2000). Therefore, we conducted phylogenetic and molecular dating analyses of *rbc*L sequences of Crypteroniaceae and all other families of Myrtales with the following aims: (1) to test the circumscription of Crypteroniaceae and identify their sister group; (2) to infer the age of Crypteroniaceae based on different molecular dating approaches; and (3) to use the chronological and phylogenetic data to elucidate the biogeographic history of Crypteroniaceae and related families.

MATERIALS AND METHODS

Molecular Methods

DNA was extracted from frozen or silica-gel dried leaves from single individuals of *Crypteronia paniculata* Bl., *Dactylocladus stenostachys* Oliv., and *Axinandra zeylanica* Thw., plus *Penaea mucronata* L., and *Sonderothamnus petraeus* (Barker) R. Dahlgr. (Penaeaceae) using DNEasy Plant Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturers instructions (see Table 3). *rbc*L was symmetrically amplified with the polymerase chain reaction (PCR; Mullis and Faloona 1987). PCR started with 2.5 min at 95°C, proceeded with 30 cycles of 1 min at 94°C, 1 min at 48°C, 2

TABLE 2. Distribution and habitat preferences of the five genera ascribed to Crypteroniaceae by different authors. See Table 1 for references.

Genus	No. of species	Distribution	Habitat
Axinandra	4	Sri Lanka (*A. zeylanica*) Borneo and Malay Peninsula	Lowland and submontane rain-forests
Crypteronia	7	Southeast Asia (S. Yunnan, Assam, Bengal, Lower Burma, Thailand, Cambodia, S. Vietnam, Malay Peninsula, Sumatra, Java, Lesser Sunda Is., Philippines, Borneo, Celebes, Moluccas, New Guinea)	Lowland and submontane rain-forests below about 1300 m
Dactylocladus	1	Borneo	Lowland peat swamp forests
Rhynchocalyx	1	South Africa (S. Natal and N. Transkei)	Moist tropical forests
Alzatea	1	Costa Rica, Panama, Peru, Bolivia	Tropical submontane forests

TABLE 3. Species names, sources, and GenBank accession numbers of the newly generated *rbc*L sequences. Herbaria acronyms: Z, Zurich; BOL, Bolus (University of Cape Town).

Species name	Voucher or source	GenBank numbers
Sonderothamnus petraeus (Barker) R. Dahlgr.	J. Schönenberger 362 (Z, BOL)	AY078154
Penaea mucronata L.	J. Schönenberger 354 (Z, BOL)	AY078155
Crypteronia paniculata Bl.	M.W. Chase 1235 (Bogor Botanic Garden VIII.B.67)	AY078153
Axinandra zeylanica Thw.	N. Gunatilleke s.n. (Sinharaja MAB reserve, Sri Lanka)	AY078157
Dactylocladus stenostachys Oliv.	K.M. Wong s.n. (Sandakan, Sabah)	AY078156

min at 72°C, and ended with an extension of 7 min at 72°C. Before sequencing, the PCR products were run on a 1% agarose gel, sliced out and purified with the QIAquick PCR Purification Kit (Qiagen, Basel, Switzerland). Sequencing reactions were performed with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Rotkreuz, Switzerland), according to the manufacturers instructions, and were purified with MicroSpin G-50 Columns (Amersham Pharmacia Biotech, Duebendorf, Switzerland) and sequenced on an ABI Prism 377 DNA Sequencer (Perkin Elmer, Rotkreuz, Switzerland). Partial sequences were assembled using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI) and were aligned manually (no indels needed). GenBank accession numbers and sources of material for the five newly generated *rbc*L sequences are provided in Table 3. The other *rbc*L sequences used in the analyses are from Conti et al. (1996), except for *Olinia*, *Medinilla*, and *Mouriri*, which are from Clausing and Renner (2001).

Phylogenetic Analyses

Phylogenetic analyses were performed in PAUP 4.0b8 (Swofford 2000). The first dataset comprised a total of 48 taxa, representing all families ascribed to Myrtales and nine taxa used for global outgroup comparison (Maddison et al. 1984): *Acer*, *Ailanthus*, *Tropaeolum*, *Capparis*, *Limnanthes*, *Carica*, *Gossypium*, *Tilia*, *Sarcolaena* (Conti et al. 1996, 1997). This larger dataset was subjected to maximum-parsimony (MP) analyses aimed at defining the circumscription of Crypteroniaceae and their sister group. Most-parsimonious trees were found using a heuristic search, with 100 random taxon additions, tree bisection reconnection (TBR), and steepest descent activated. All characters were unordered and equally weighted. Tree statistics were calculated both for all characters and for informative characters only. Statistical support for each branch of the most parsimonious trees was assessed with a bootstrap analysis conducted on the original data matrix by generating 100 pseudoreplicates and by using the above mentioned heuristic search strategy.

Once the placement of Crypteroniaceae within Myrtales was clarified, a smaller dataset of 13 taxa, including only Crypteroniaceae and their phylogenetic neighbors was used to perform the computationally intensive maximum-likelihood (ML) analyses aimed at reconstructing the biogeographic history of Crypteroniaceae. An initial Fitch parsimony analysis with Branch-and-Bound provided starting trees for the estimation of ML parameters under 56 different evolutionary models using Modeltest 3.06 (Posada and Crandall 1998). The optimal model was selected using a likelihood ratio test (Felsenstein 1981; Huelsenbeck and Rannala 1997) and parameters estimated by the best evolutionary model were then used in a ML heuristic search performed with 100 random addition sequences, TBR, and steepest descent activated. Trees were rooted as suggested by the initial, broader analysis. Support for different clades under ML was estimated by 1000 bootstrap replications using the fast-heuristic search option in PAUP 4.0b8 (Swofford 2000). The distribution of variable characters in the 13-taxon dataset according to codon position was established by alternatively excluding first and second or third codon positions, respectively.

Molecular Dating

To evaluate whether the *rbc*L sequences in the smaller, 13-taxon dataset evolved in a clocklike fashion, a likelihood ratio (LR) test was performed by comparing the scores of ML trees with and without the clock (Felsenstein 1981; Sanderson 1998). The LR was calculated as $2(\ln L_{\text{clock}} - \ln L_{\text{no clock}})$ (Nei and Kumar 2000) and was assumed to be distributed as a χ^2 with the number of degrees of freedom (df) equal to the number of taxa minus two, that is, 11 (Sanderson 1998). To estimate absolute substitution rates, it is necessary to fix at least one node, which is usually done by reference either to the fossil record (paleobotanic dating) or to known vicariance events (geologic dating; Hillis et al. 1996; Sanderson 1998). Either approach can establish only the minimum ages at the calibration point, possibly resulting in an underestimation of divergence times (Tavaré et al. 2002). As reliable fossils of Crypteroniaceae have not yet been discovered (Anderson and Muller 1975; Muller 1975, 1981), we used a calibration point in the outgroup: fossil leaves of Melastomataceae dated at 53 million years ago (mya) (calibration 1; Renner et al. 2001). We also used geologic dating based on the break-up of western Gondwana using both the onset (120 mya: calibration 2) and the end (90 mya: calibration 3) of the South American-African split (Scotese and McKerrow 1990; McLoughlin 2001). Other studies have recently used geologic calibration for molecular-based dating estimates, for example, in *Phylica* (Richardson et al. 2001), Laurales (Renner et al. 2000), ranid frogs (Bossuyt and Milinkovitch 2001), and ratite birds (Cooper et al. 2001).

To gain some insight into the relative performance of different molecular dating methods, the ages estimated using the clock-based ML trees were compared with the times estimated from clock-dependent Langley-Fitch (LF) trees (Langley and Fitch 1974) and clock-independent penalized-likelihood (PL) analysis (see below; Sanderson 2001, 2002; Table 4). In the case of ML, absolute substitution rates were estimated by dividing the sum of the branch lengths from the calibrated node to the tips (i.e., the nodal depth) by the cal-

TABLE 4. Divergence time estimates (expressed in million years ago, rounded to the nearest integer) for the nodes marked on the trees of Figures 1 and 3 using maximum-likelihood (ML), Langley-Fitch (LF), and penalized-likelihood (PL) dating methods (see Methods) and according to three independent calibrations: 1, fossil leaves of Melastomataceae (Node E: 53 million years ago); 2, initial separation of South America and Africa (Node C: 120 million years ago); 3, final separation of South America and Africa (Node C: 90 million years ago). The range of ages estimated for each node using the three different calibration points are reported below each node. Numbers in bold correspond to those used for calibrations.

		Method		
Node	Calibration	ML	LF	PL
A	1	23	26	47
	2	70	59	60
	3	54	44	45
	Range	47	33	15
B	1	50	68	110
	2	150	151	141
	3	115	113	106
	Range	100	83	31
C	1	40	54	94
	2	**120**	**120**	**120**
	3	**90**	**90**	**90**
	Range	80	66	26
D	1	90	92	101
	2	270	204	130
	3	207	153	97
	Range	180	112	33
E	1	**53**	**53**	**53**
	2	160	117	68
	3	123	88	51
	Range	107	64	15

ibration time (Page and Holmes 1999). Ages were placed on other nodes by dividing their nodal depth by the absolute substitution rate (Sanderson 1998). Similarly, in LF trees, the calibration date was divided by the relative age of the corresponding constraint node, yielding a relative substitution rate that was multiplied by the relative ages of the other nodes to obtain absolute ages (Sanderson 1998, 2001). To estimate the position of the root node within the basal branch in the LF and PL analyses, an outgroup, *Heteropyxis*, was included. However, this taxon was removed before performing further calculations (Sanderson 1998, 2001).

Penalized likelihood, implemented in the software r8s (Sanderson 2001), is a semiparametric smoothing method that estimates relative branching time without assuming a molecular clock (Sanderson 2002). Like nonparametric rate smoothing (NPRS; Sanderson 1997), PL assumes autocorrelation of substitution rates and attempts to minimize rate changes between ancestral/descendant branches on a tree. However, NPRS approaches have been proved to overfit the data, allowing too much rate variation and thereby losing predictive power (Sanderson 2002). Penalized likelihood aims at combining the statistical power of parametric methods, which use parameter-saturated models of rate evolution, with the robustness of nonparametric methods, by assigning penalties that constrain rate changes between sequential branches in a tree. Branches are permitted to have different rates of molecular evolution, but a penalty is assessed when rates change from ancestral to descendant branches. Smoothing levels, λ, can vary from very small, in which essentially every branch of the tree is free to have a different substitution rate, reflecting extreme non clocklike behavior, to very large, in which parameters converge to the clocklike model. The key to the application of penalized likelihood is to find an objective method to choose the optimal smoothing level. The used method is based on a data-driven cross-validation procedure that sequentially prunes taxa from the tree, estimates parameters from the submatrix for a given smoothing value, predicts the data that were removed by using the estimated parameters, and calculates the χ^2 error associated with the difference between predicted and observed data of the removed submatrix. The optimal smoothing level corresponds to the lowest χ^2 error (Sanderson 2002).

Because PL analyses are computationally very time-consuming, 95% confidence intervals of nodal depth (hence age estimates) were calculated only for selected nodes. One hundred bootstrap pseudoreplicates were generated from the *rbc*L data matrix using SEQBOOT in Phylip 3.5c (Felsenstein 1993). While keeping the tree fixed, for each pseudoreplicate the nodal depth of the node in question was estimated by ML with the preferred model of molecular evolution (Sanderson 1997). For each node, the standard deviation s and mean age μ were calculated on a distribution of 100 ages obtained by bootstrapping the *rbc*L data matrix. Confidence intervals were defined by $\mu \pm 2s$.

As high smoothing levels in PL analysis reflect constant substitution rates across different branches of a tree, one would expect that the likelihood ratio test (Felsenstein 1981) would not reject the clock assumption in datasets that yield high values for λ-optimal. Conversely, one would expect that, for datasets with small λ-optimal values, the likelihood ratio test would reject the clock assumption. To explore the effects of taxon sampling on this expected relationship between λ-optimal and the degree to which a clock is supported/rejected (as indicated by the *P*-value returned from the clock versus nonclock likelihood ratio test, described above) we calculated λ-optimal and *P*-values for six progressively larger datasets, starting from an 8-taxon dataset that only included taxa from the more ultrametric part of the tree, based on visual inspection, (i.e., Crypteroniaceae and their sister western Gondwanan clade), and sequentially adding one taxon at a time (*Mouriri*, *Rhexia*, *Medinilla*, *Tibouchina*, and *Osbeckia*) from the more additive part of the tree, up to 13 taxa (see Fig. 2a and Table 5).

RESULTS

Phylogenetic Analyses

All *rbc*L sequences were 1428 base pairs (bps) long and could be aligned without gaps. For the 48-taxon dataset there were 294 parsimony-informative characters. For this dataset heuristic searches identified a single island of 48 MP trees of 1232 steps when all characters were included (consistency index [CI] = 0.475; retention index [RI] = 0.632). When only informative characters were included, tree length (L′) was 1064 and the consistency index (CI′) was 0.392. The strict consensus tree (see Fig. 1) shows the following important results: (1) *Crypteronia*, *Axinandra*, and *Dactylocladus* form a monophyletic group, with a bootstrap value (BS)

TABLE 5. Comparisons between optimal smoothing values (λ) estimated in penalized-likelihood analyses and *P*-values obtained from likelihood ratio (LR) tests. The smallest dataset included eight taxa (Crypteroniaceae sensu stricto plus their western Gondwanan sister clade; see Fig. 1); the taxon added in each round is reported in parentheses in the first column. The likelihood ratios ($2[\ln L_{clock} - \ln L_{no\ clock}]$:LR) are given in the third column (with degrees of freedom in parentheses) and the corresponding *P*-values in the fourth (based on a χ^2 distribution).

Number of taxa (taxon added)	λ	LR (df)	*P*-value
8	10^6	9.50 (6)	0.15
9 (*Mouriri*)	0.00	8.58 (7)	0.28
10 (*Rhexia*)	0.00	10.88 (8)	0.21
11 (*Medinilla*)	0.00	15.69 (9)	0.07
12 (*Tibouchina*)	0.01	23.26 (10)	0.01
13 (*Osbeckia*)	0.01	21.66 (11)	0.03

of 99% (2) *Alzatea* (Alzateaceae), *Rhynchocalyx* (Rhynchocalycaceae), *Sonderothamnus* (Penaeaceae), *Penaea* (Penaeaceae), and *Olinia* (Oliniaceae) form a clade with a BS of 82%; (3) the aforementioned two clades are sister to each other with a BS of 82%; and (4) this latter clade, in turn, is sister to Melastomataceae, with a BS of 94%. Partial chloroplast DNA sequences of seven noncoding regions from *Crypteronia paniculata* only (J. Schönenberger, unpubl. data) corroborate its sister relationship to the clade comprising *Alzatea*, *Rhynchocalyx*, *Olinia*, and Penaeaceae.

Based on the results of the 48-taxon dataset, further analyses were conducted on a 13-taxon dataset comprising all accessions from Melastomataceae, Crypteroniaceae sensu lato, Penaeaceae, and Oliniaceae. For this dataset, nested likelihood ratio tests showed that the best model for *rbc*L evolution is the general time reversible (GTR; Rodríguez et al. 1990) model with gamma (Γ) shape distribution (Yang 1993). The following parameters were calculated based on this model: relative rate of substitution between A ↔ C = 0.9689, A ↔ G = 1.4352, A ↔ T = 0.3240, C ↔ G = 0.4803, C ↔ T = 1.6714; Γ shape parameter, α = 0.6816. By using these parameters, an ML tree was obtained with a log-likelihood score of −3478.6277 (see Fig. 2). This tree is entirely consistent with the MP tree obtained for the larger dataset, but additionally suggests a sister-group relationship between the South American *Alzatea* and an African clade comprising *Rhynchocalyx*, *Olinia*, *Sonderothamnus*, and *Penaea* (both Penaeaceae). By enforcing the molecular clock we obtained an ML tree with a score of −3489.4621 and a topology that was identical to that of the nonclock tree (except for the relationships of *Medinilla* and *Rhexia* in the outgroup), rooted as predicted by the 48-taxon analysis (see Fig. 2b). Comparison with the nonclock model suggested that a molecular clock could be rejected (LR = 21.67, df = 11, *P* = 0.03). The distribution of variable characters in the 13-taxon dataset was as follows: when all codon positions were included, there were 159 variable characters; when only third codon positions were included, there were 103 variable characters; when only first and second codon positions were included, there were 56 variable characters in the *rbc*L sequences.

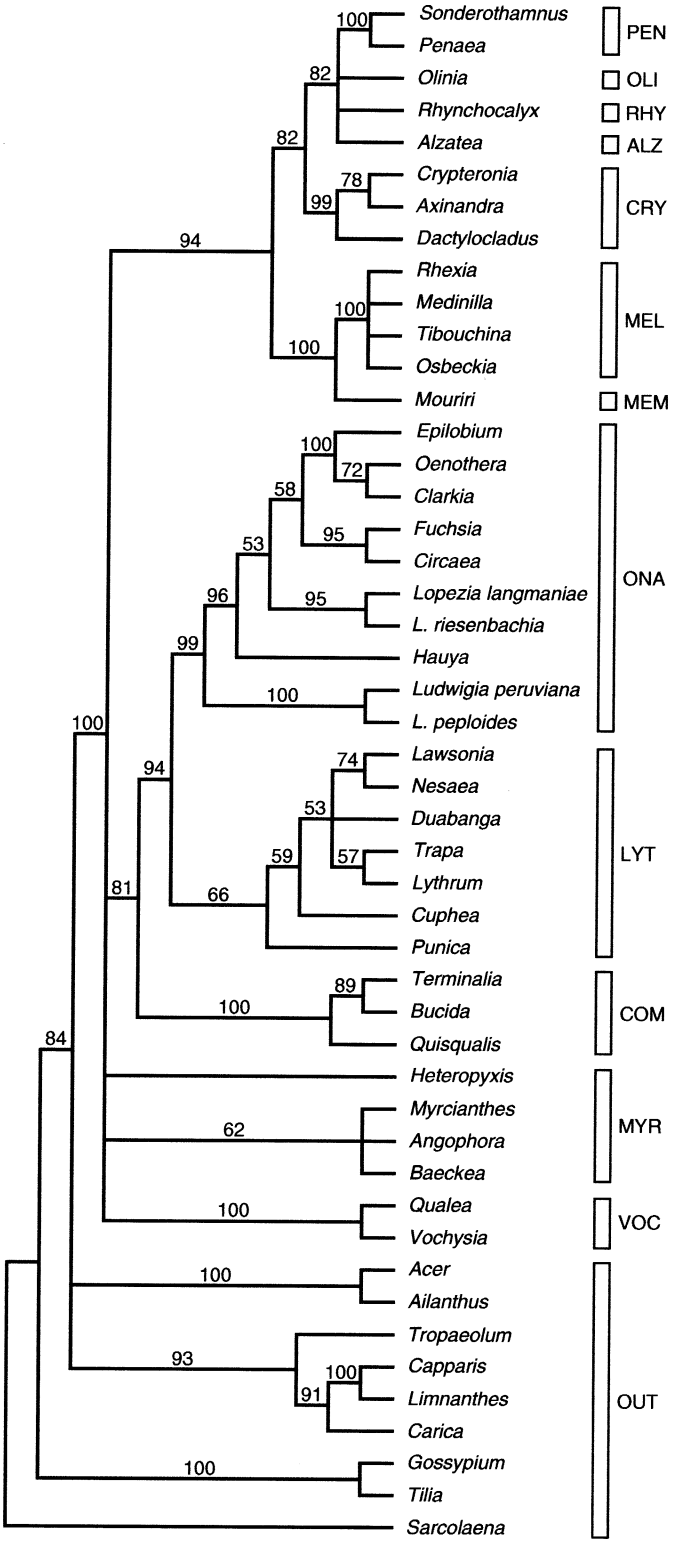

FIG. 1. Strict consensus bootstrap tree resulting from maximum parsimony (MP) analyses of the 48-taxon *rbc*L dataset. Bootstrap values >50% are reported above the branches. Familial affiliations in Myrtales are reported to the right of the tree: PEN, Penaeaceae; OLI, Oliniaceae; RHY, Rhynchocalycaceae; ALZ, Alzateaceae; CRY, Crypteroniaceae sensu stricto; MEL, Melastomataceae; MEM, Memecylaceae; ONA, Onagraceae; LYT, Lythraceae; COM, Combretaceae; MYR, Myrtaceae; VOC, Vochysiaceae; OUT, Outgroup.

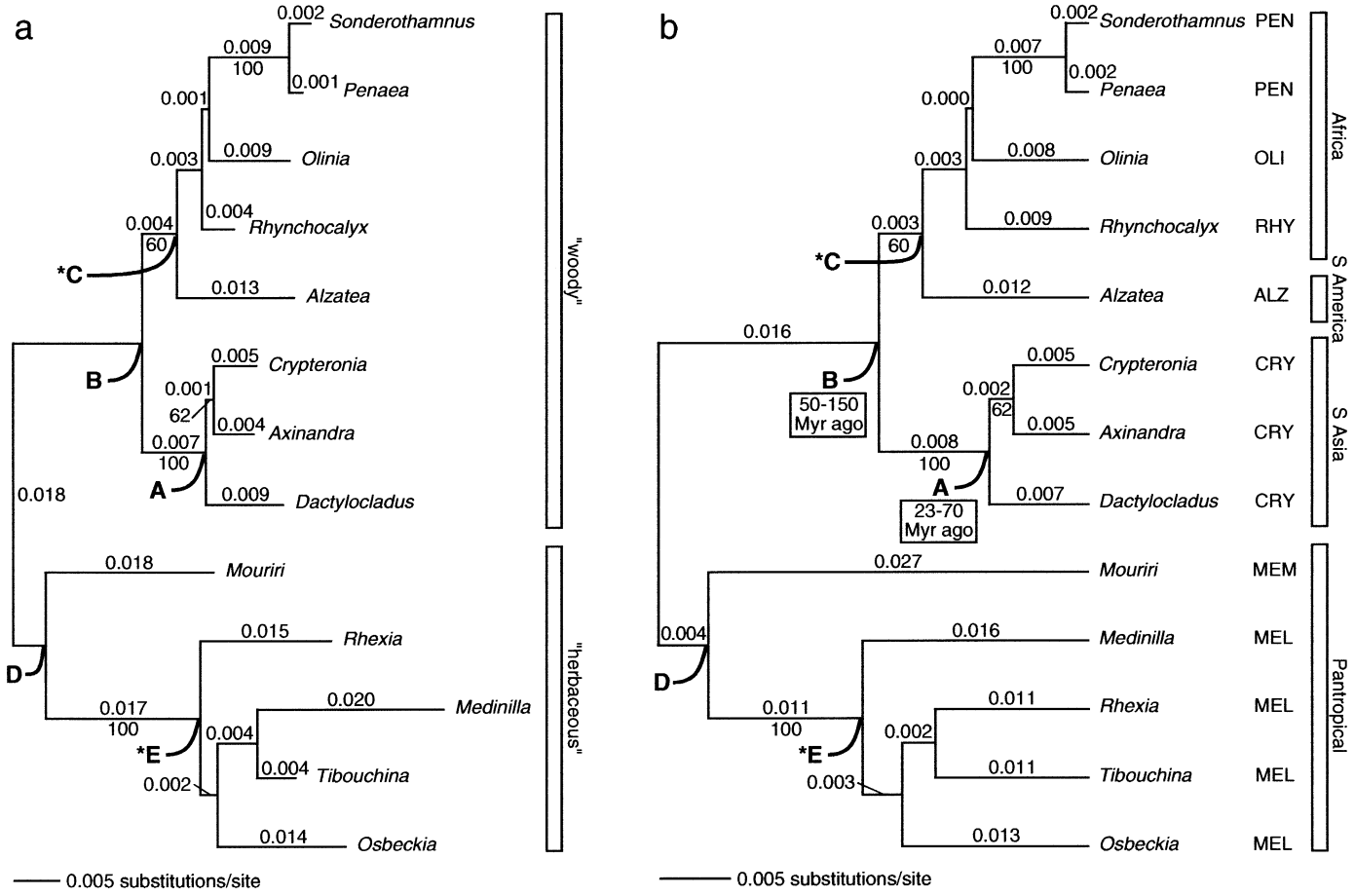

FIG. 2. Maximum likelihood (ML) trees without (a) and with (b) a molecular clock enforced. Bootstrap values are reported below and branch lengths above the branches. Familial affiliation is given after taxon name (as in Fig. 1) and distribution to the right of the clock tree (b). ''Woody'' refers to the more ultrametric and ''herbaceous'' to the more additive parts of the tree (see Discussion). Nodes of interest: A, diversification of Crypteroniaceae crown group; B, origin of Crypteroniaceae stem lineage; C, diversification of the western Gondwanan crown group; D, origin of Melastomataceae stem lineage; E, diversification of Melastomataceae crown group. Nodes used for calibration are marked by an asterisk. The numbers within the frames in the clock tree (b) indicate the range of ML datings across the three calibrations for nodes A and B (see Table 4).

Molecular Dating

Despite the narrow rejection of a molecular clock, we proceeded to estimate node ages with maximum likelihood under the clock assumption so as to compare them to ages obtained by two other methods, Langley-Fitch and penalized-likelihood. Three different calibration times were used to calculate substitution rates based on branch lengths of the ML tree with a clock enforced (see Table 4 and Fig. 2). When fossil leaves of Melastomataceae were used to constrain node E (calibration 1) a substitution rate of 3.02×10^{-4} substitutions per site per million years (SSMY) was obtained. When the beginning of the split between South America and Africa was used to constrain node C (calibration 2), SSMY were estimated to be 1.00×10^{-4}. When the end of the split between South America and Africa was used (calibration 3), SSMY were estimated to be 1.33×10^{-4}. Converting nodal depths of four key nodes into ages, the fastest estimated substitution rate produced the youngest age estimates and the slowest substitution rate produced the oldest age estimates (see Table 4; Fig. 2).

When using fossil calibration on node E (calibration 1), PL (see Fig. 3) suggested a much older age for nodes A–C than the other methods, whereas the estimated age of node D was slightly older (see Table 4). However, when using geologic calibrations on node C (calibrations 2 and 3), PL produced age estimates that were intermediate between or slightly younger than those obtained with ML and LF for nodes A and B and much younger for nodes D and E. All ages estimated for node A (diversification of Crypteroniaceae), regardless of calibration point or dating method, fall within the confidence interval (14–90 mya) obtained by bootstrapping with PL (see Figs. 2, 3, and Table 4). For node B (origin of Crypteroniaceae), the mean age of the 100 PL bootstrap replicates was 116 mya, with a standard deviation of ±24 million years, and a 95% confidence interval between 68 and 164 mya. All ages estimated for this node, except the ML dating obtained with calibration 1, fall within this confidence interval (see Table 4).

Comparisons among the λ smoothing values estimated in PL and the *P*-values obtained from the likelihood ratio test of a clock across a range of subsampled datasets are reported in Table 5.

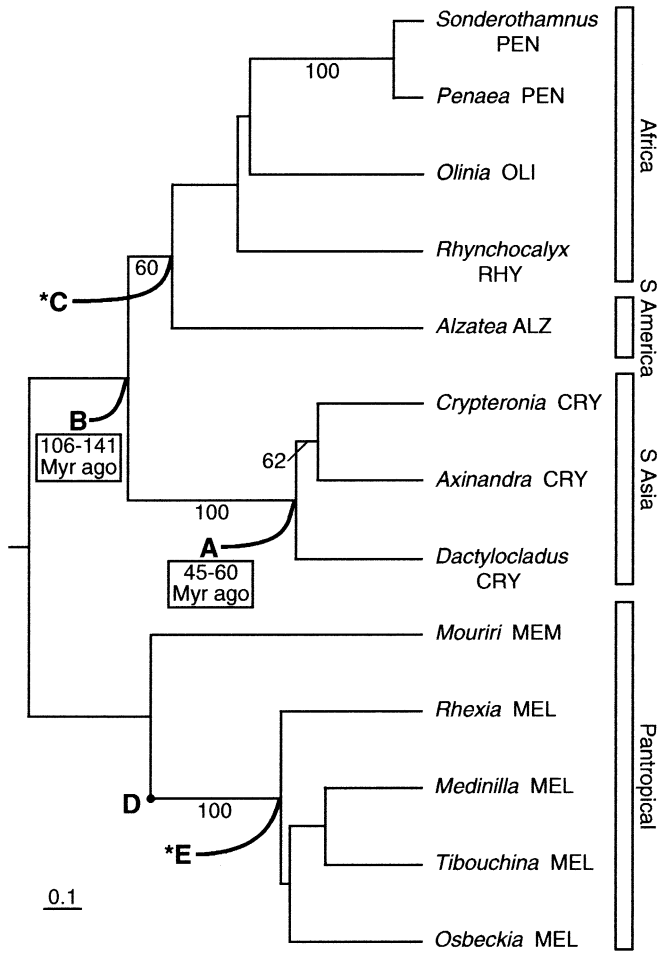

FIG. 3. Penalized-likelihood (PL) tree with branches proportional to time. Branch support, marked nodes, familial affiliation, and geographic distribution as in Figure 2 caption. The numbers within the frames indicate the range of PL datings across the three calibrations for nodes A and B (see Table 4).

DISCUSSION

Rates of rbcL Evolution and Lineage Effects

An interesting question in any molecular dating study is whether the rates of nucleotide substitution vary systematically across lineages. Obviously, these comparisons should be approached cautiously, because they will be influenced by methods of analysis and the models of molecular evolution assumed. In the case of our 13-taxon *rbc*L dataset, rates of nucleotide substitution were obtained from branch lengths calculated with the GTR + Γ model (Rodríguez et al. 1990; Yang 1993). The low value of the Γ shape parameter ($\alpha = 0.6816$) for the *rbc*L sequences shows that there is great among-site rate heterogeneity, which accord with previous data showing that substitutions are heavily concentrated in the third codon position of *rbc*L (Chase and Albert 1998). Our own analysis of the distribution of variable characters according to codon position confirms that almost twice as many variable characters (103) are found in the third codon position, as compared to first and second codon positions together (56).

The clock-based estimates of *rbc*L substitution rates vary between 3.02×10^{-4} SSMY, obtained with fossil calibration on node E, and $1.33–1.00 \times 10^{-4}$ SSMY, obtained with geologic calibrations on node C. The fastest rate is closer to the rate calculated for the shrub *Gossypium* (2.5×10^{-4}; Wendel and Albert 1992), whereas the two slower rates are closer to those obtained for trees in Atherospermataceae (0.9×10^{-4}, 2.4×10^{-4}; Renner et al. 2000) and in a survey of woody seed plant families (1.0×10^{-4}; Albert et al. 1994). These comparisons with *rbc*L rates in other plant groups suggest that differences of rates in our *rbc*L dataset might be caused by lineage effects (Sanderson 2001). Specifically, the two slower rates were obtained when constraining node C, which represents the common ancestor of five woody genera, ranging from shrubs (*Sonderothamnus*, *Penaea*) to treelets (*Olinia*), and trees (*Rhynchocalyx* and *Alzatea*). On the other hand, the faster rate was obtained when constraining node E, the common ancestor of three mostly herbaceous genera (*Osbeckia*, *Medinilla*, and *Rhexia*) and one shrubby genus (*Tibouchina*). The difference between ''herbaceous'' and ''woody'' parts of the ML tree is also obvious by visual inspection of the unconstrained ML tree (see Fig. 2a), which shows longer branches for the herbaceous taxa and shorter branches for the woody taxa. These results are consistent with previous reports of a slowdown in the evolutionary pace of woody versus herbaceous taxa, observed, for example, in palms versus grasses (Gaut et al. 1992; Gaut 1997) and in the woody *Fuchsia* versus its herbaceous sister *Circaea* (Onagraceae; Conti et al. 1993). These observations and the fact that almost two-thirds of all variable characters in the 13 *rbc*L sequences occur in the third codon position, implying that most substitutions are synonymous, support the conclusion that rates of substitution at silent sites might be especially sensitive to generation time (Wu and Li 1985; Gaut et al. 1992; Ohta 1995), in accordance with a neutralist interpretation of rate variation (Page and Holmes 1999).

Variation in evolutionary rates was also reflected in the different ages estimated for each node under clock-dependent (ML and LF) and clock-independent (PL) approaches (see Table 4). Penalized likelihood produced dating estimates for each node that were within a much narrower range than those obtained with the two clock-based approaches. Accordingly, the age range for the diversification of Crypteroniaceae (node A) under PL was only 15 million years, while it was 33 million years under LF, and 47 million years under ML. Thus, PL provided reciprocal corroboration among the ages estimated with paleobotanic and geologic calibrations. For example, when using fossil leaves of Melastomataceae at 53 mya, we obtained a dating estimate for node C of 94 mya, within the range (120–90 mya) of the geologic estimates for the splitting of western Gondwana (Scotese et al. 1988; McLoughlin 2001). Similarly, when using geologic calibrations on node C to estimate the age of node E, we obtained dates of 68 and 51 mya, which bracketed the age of fossil leaves of Melastomataceae at 53 mya (see Table 4). Since the age estimated for node E (51 mya) using the final separation of western Gondwana (90 mya) as a calibration point is slightly younger than the age (53 mya) attributed to fossil leaves of Melastomataceae, it seems reasonable to suppose that the

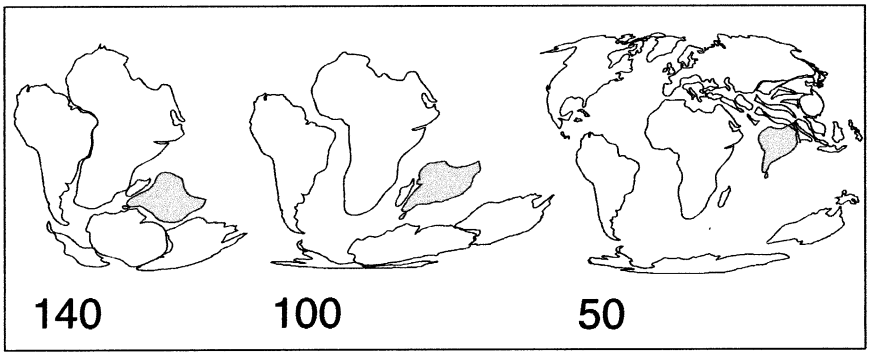

FIG. 4. Paleogeographic reconstructions of Gondwanan plates during the Early and Middle Cretaceous and the Early Tertiary; numbers refer to millions of years ago. Maps modified from Scotese (1997).

origin of the western Gondwanan clade predated the final separation of the supercontinent.

If different rates of evolution characterize different lineages, this should also be reflected in the degree to which the molecular clock assumption is accepted/rejected by the LR test as taxon sampling varies (Sanderson and Doyle 2001). Specifically, we expected that, if one kept only the taxa from the more ultrametric part of the tree, *P*-values from the LR test would be higher, as would λ-optimal values. Accordingly, as taxa were introduced that perturbed rate constancy, we expected that *P*-values would dip below $P = 0.05$, our chosen critical value, and λ-optimal values would likewise drop. The effects of taxon sampling on P and λ-optimal values are summarized in Table 5. The smallest, 8-taxon dataset comprised only Crypteroniaceae and their western Gondwanan sister clade, characterized by low and relatively constant rates (see Fig. 2a). As expected, the 8-taxon dataset produced a very high λ-optimal value and a LR value that did not cause the clock assumption to be rejected. The progressive addition of taxa immediately caused a sharp drop of optimal smoothing values to 0 (with 9-, 10-, and 11-taxon datasets) or 0.01 (with 12- and 13-taxon datasets), as one would expect in cases of rate variation across branches (see Table 5). However, the LR test did not reject the clock assumption for the 9-, 10-, and 11-taxon datasets, and returned significant values only for the 12- and 13-taxon datasets. A direct relationship between the number of taxa included in the analysis and the power of the LR test has already been proposed (Sorhannus and Van Bell 1999). Our comparisons between λ-optimal and *P*-values suggest that perhaps the LR test is not sufficiently sensitive to detect localized deviations from clocklike evolution and that λ-optimal values may better reflect even small rate variations. Our results contradict previous suggestions that global LR tests represent a conservative approach towards detecting rate constancy (Sanderson 1998). In other words, the potential for committing Type-II error with the LR test may be higher than previously thought and therefore the null hypothesis of clocklike evolution should be considered with caution, even if it is not statistically rejected by the LR test (Ott 1988). Concerns about the power of LR test had already been raised in simulation experiments that explored the performance of the LR test under different conditions of taxon sampling (Sorhannus and Van Bell 1999).

Phylogenetic Relationships and Biogeography

Our phylogenetic analyses of *rbc*L sequence data, based on both maximum-parsimony and maximum-likelihood criteria, are congruent with a narrow circumscription of Crypteroniaceae comprising the three southern and Southeast Asian genera *Axinandra*, *Crypteronia*, and *Dactylocladus* (simply Crypteroniaceae for the rest of this discussion; see Figs. 1 and 2), in agreement with the view of most recent authors (see Table 1). Our analyses further suggest that Crypteroniaceae sensu lato, as delimited by van Beusekom-Osinga and van Beusekom (1975), are nonmonophyletic. Furthermore, our results strongly support the sister relationship between Crypteroniaceae and a clade that comprises the South American genus *Alzatea* and the three African taxa *Rhynchocalyx* (a monospecific genus), *Olinia* (a genus with seven to nine species; Mújica and Cutler 1973), and Penaeaceae, a family that includes seven genera with 23 currently recognized species (Dahlgren and Thorne 1984; Dahlgren and van Wyk 1988; see Figs. 1 and 2). These results corroborate previous hypotheses proposed on embryological grounds (Tobe and Raven 1983, 1987a, b) and in a morphological cladistic analysis of Myrtales (Johnson and Briggs 1984), while contradicting the close relationship of Crypteroniaceae with Melastomataceae or Lythraceae suggested by anatomical (Mentink and Baas 1992) and leaf histological characters (Keating 1984; see Table 1).

Any discussion on the origin of a monophyletic group should make a distinction between the origin of the stem lineage and the origin of the crown group (Sanderson and Doyle 2001). In the case of Crypteroniaceae, their stem lineage can be identified as the lineage that split off from their western Gondwanan sister clade (node B, see Figs. 2 and 3), whereas the crown group comprises all living descendants (*Crypteronia*, *Axinandra*, and *Dactylocladus*) of the most recent common ancestor (node A, see Figs. 2 and 3). The *rbc*L phylogeny is fully concordant with the splitting order of Gondwana, implying that the fragmentation of Gondwanan landmasses from the Early to the Late Cretaceous, possibly aided by short-distance, over-water dispersal, shaped the biogeographic history of these taxa (see Fig. 4; Scotese and McKerrow 1990; Scotese 1997; McLoughlin 2001). This hypothesis is congruent with the time suggested for the diversification of angiosperms (Sanderson and Doyle 2001; Wick-

ström et al. 2001) and the proposed western Gondwanan origin of Myrtales (Raven and Axelrod 1974; Johnson and Briggs 1984). Although Raven and Axelrod (1974) initially suggested a Laurasian origin for Crypteroniaceae, Tobe and Raven (1984, p. 115) viewed the biogeography of Crypteroniaceae, Oliniaceae, Penaeaceae, Rhynchocalycaceae, and Alzateaceae as indicating a Gondwanan origin: ''Such a distribution of related families, as well as the many distinctive features that they possess, certainly suggest the fragmentation of a relatively ancient ancestral line almost certainly dating to the Cretaceous.'' Gondwanan vicariance has been proposed to explain the distributions of several other angiosperm groups, including Liliales (Vinnersten and Bremer 2001) and Atherospermataceae (Renner et al. 2000).

A vicariant hypothesis, with the origin of the stem lineage of Crypteroniaceae in Gondwana, provides the most parsimonious explanation without invoking any unnecessary long-distance dispersal events. If Gondwanan vicariance were ruled out, one would need to assume at least two transoceanic dispersal events to account for the current geographical distribution of Crypteroniaceae and its allies. The morphology of fruits and seeds and their potential to disperse should also be discussed in this context. Even though one should not assume that the same character states of extant taxa were present in extinct ancestors, it is noteworthy that all extant members of Crypteroniaceae and their western Gondwanan sister clade have capsular fruits with very small seeds (Meijer 1972), with the exception of Oliniaceae, which have fleshy fruits (Rao and Dahlgren 1969). Therefore, it is most parsimonious to suggest that the most recent common ancestor (MRCA) of these five families (node B, see Figs. 2 and 3) was characterized by capsular fruits and small seeds (Meijer 1972). Given these inferred fruit characteristics of the MRCA and the age of the stem lineage supported by PL analyses (106–141 mya; node B; see Fig. 3), short- to medium-dispersal of the Crypteroniaceae stem lineage from Africa to the Indian plate, rafting along the African coast from the Middle Cretaceous to the early Paleogene (Morley 2000; Scotese 1997; see below), seems more likely than long-distance, transoceanic dispersal.

If we accept the Gondwanan origin of the Crypteroniaceae stem lineage between 106 and 141 mya (node B; Fig. 3), then we need to propose a plausible migration route by which Crypteroniaceae reached their current distribution. At present, Crypteroniaceae occur primarily in Southeast Asia, with only one species, *Axinandra zeylanica*, in Sri Lanka (see Table 2). The question then arises as to whether any of these two regions were ever connected with Gondwana in a time frame that is compatible with our molecular estimates for the age of Crypteroniaceae. Most of Southeast Asia was formed by several continental slivers that broke off from Northern Gondwana sometime during the late Paleozoic to early Mesozoic (Metcalfe 1996). Therefore, paleogeographic data do not favor the possibility of direct dispersal from Gondwana to Southeast Asia within a time frame that is compatible with angiosperm evolution (Axelrod 1971; Raven and Axelrod 1974; Raven 1979; McLoughlin 2001; Sanderson and Doyle 2001; Wikström et al. 2001). Instead it is necessary to invoke rafting on India followed by range expansion within tropical Asia.

The India-Seychelles-Madagascar (Deccan) plate separated from eastern Gondwana in the Early Cretaceous, approximately 130 mya (Scotese et al. 1988; McLoughlin 2001). Following this separation, the plate moved northward, remaining close to the African coast, until the India-Seychelles block rifted from Madagascar about 90 mya. Thereafter, the Indian block, including Sri Lanka, continued its journey northeast until it collided with Asia in the early Tertiary (see Fig. 4; Beck et al. 1995; Storey et al. 1995). During its geologically rapid rafting towards Asia, India underwent remarkable latitudinal, hence climatic and vegetational changes (McLoughlin 2001; Morley 2000). Furthermore, extensive volcanism, approximately 65 mya, erased many taxa from central western India (Officer et al. 1987). Upon impact with Asia in the Early Tertiary, the uplift of the Himalayan chain and subsequent Late Tertiary aridification further contributed to the progressive impoverishment of autochthonous eastern Gondwanan and allochtonous African elements from the Indian biota, with the exception of refugial areas in southwestern India and Sri Lanka (Raven and Axelrod 1974; Gulleria 1992; Morley 2000).

To establish whether the times of origin and diversification of Crypteroniaceae are compatible with the out-of-India hypothesis, we used ages estimated with the PL approach, because all lines of evidence discussed above imply significant rate variation across the *rbc*L tree, making clock-dependent methods unreliable (Wray 2001). Penalized-likelihood estimates for the Crypteroniaceae stem lineage (node B; see Fig. 3) ranged between 106 and 141 mya (see Table 4), a time at which India was sufficiently close to Africa to receive western Gondwanan elements by short-distance dispersal, perhaps aided by island hopping via Madagascar and island chains that possibly existed between Africa and Madagascar (see Fig. 4; Scotese et al. 1988; Morley 2000; McLoughlin 2001). Our analyses further suggested that the Crypteroniaceae crown group (node A; see Fig. 3) began radiating between 45 and 60 mya, implying that the initial diversification of the family occurred in India prior to the impact with Asia (Beck et al. 1995; Storey et al. 1995). Since direct communication between India and the rest of Asia did not start until the Paleogene, and dispersal of Crypteroniaceae to Southeast Asia presumably required a few million years, it is not surprising that the only fossil pollen tentatively assigned to Crypteroniaceae, specifically the *Dactylocladus*-type from Borneo, would be dated to the Upper Miocene (Anderson and Muller 1975; Muller 1975, 1981).

If India, as we have proposed, served as a ''Noah's Ark'' (Axelrod 1971; McKenna 1973; Morley 2000) for Crypteroniaceae, one might wonder why this family has virtually disappeared from the Indian subcontinent, with the notable exception of *Axinandra zeylanica* in Sri Lanka. This pattern may be explained by the massive extinction of India's Gondwanan elements caused by Late Cretaceous/Early Tertiary climatic changes and limited survival in refugial areas, especially in Sri Lanka (Raven and Axelrod 1974; Bande and Prakash 1986; Ashton and Gunatilleke 1987; Morley 2000; Sharma 2000). Similarly, Southeast Asia, where *Crypteronia* and *Dactylocladus* exist, is another region that has long been recognized as a refugium where the equable oceanic conditions allowed tropical lineages to escape extinction (Bande

and Prakash 1986; Morley 2000). Recently, Sri Lanka and Southeast Asia have been identified as two of 25 worldwide biodiversity hotspots (Myers et al. 2000); their fundamental importance in elucidating the evolutionary and biogeographic history of the Asian flora strengthens the case for their conservation.

To summarize, the inferred phylogenetic relationships match the order of Gondwanan splitting and molecular dating analyses support a Cretaceous origin for the Crypteroniaceae stem lineage. Therefore, it seems reasonable to propose that Gondwanan drift played an essential role in the biogeographic history of Crypteroniaceae and related families and specifically that India, in its northward movement from Gondwana to Asia along the African coast, served as the most likely migration route for Crypteroniaceae. This crucial role of India as a biotic link between Africa and Southern Asia has also been suggested for a few other fossil and extant plant and animal groups (Bande and Prakash 1986; Ashton and Gunatilleke 1987; Krause and Maas 1990; Bande 1992; Macey et al. 2000; Morley 2000; Bossuyt and Milinkovitch 2001; Cooper et al. 2001). An alternative route might have entailed migration of Crypteroniaceae from Africa to Europe via island hopping during the Paleocene, followed by dispersal to Southern Asia after closure of the extensive Turgai Straits in the Early Oligocene (Scotese 1997; Morley 2000). However, this potential route would have represented a very strong filter to migration, as dispersal would have had to occur across a large epicontinental ocean and vast central Asia, already characterized by very dry climatic conditions (Morley 2000). Furthermore, this alternative Eurasian route would have required that *Axinandra zeylanica* reached Sri Lanka either via long-distance dispersal from Southeast Asia or by crossing the Himalayan barrier that arose in the early Paleogene. Even though the lack of reliable fossil record for Crypteroniaceae and related families does not allow us to unquestionably settle the issue of how Crypteroniaceae attained their current distribution, the paleogeographic and paleoclimatic evidence summarized above suggests that the alternative Eurasian route represents a less parsimonious explanation for the dispersal of Crypteroniaceae than rafting on the Indian plate with survival in refugial Sri Lanka.

The ancient, relictual nature of Crypteroniaceae and related families had already been proposed on the basis of their distribution and morphology. For example, Meijer (1972) postulated a Gondwanan origin for *Axinandra*, a genus that he interpreted as being similar to the ancestor of the entire order Myrtales. Referring to the biogeographic history of *Axinandra*, Ashton and Gunatilleke (1987, p. 263) stated: ''The disjunct distribution and generalized morphology of this lowland rain forest genus suggest considerable antiquity and possible spread into Asia by way of the Deccan Plate.'' Likewise, anatomy (van Vliet et al. 1981) and pollen morphology (Muller 1975) implied an isolated taxonomic position for *Alzatea*, suggestive of an ancient Gondwanan origin (Graham 1984). The Cape floristic region of South Africa, the main center of diversity of Penaeaceae, and Natal, where *Rhynchocalyx* is endemic, have been also proposed as refugia (Raven and Axelrod 1974).

In conclusion, evidence from phylogeny, molecular dating, paleogeography, paleoclimate reconstruction, morphology, and current distribution argue for an ancient Gondwanan origin of Crypteroniaceae in the Early to Middle Cretaceous, followed by diversification on the rafting Indian plate in the Early Tertiary and subsequent dispersal to Southeast Asia. This suggests that, despite rapid latitudinal changes, massive volcanism, and dramatic orogenic events, ancient Gondwanan elements managed to survive on the Deccan plate and from there dispersed to Asia. While the idea that India served as a ''Noah's Ark'' for Gondwanan plants has been suggested previously, this study represents the first case in which it has been rigorously demonstrated using a combination of phylogenetic evidence and molecular dating.

Acknowledgments

We are grateful to P. Linder, R. Nyffeler, and the Journal Club participants of the Institute of Systematic Botany at the University of Zurich for fruitful discussions on molecular dating. We also thank N. Wikström and M. Sanderson for help with the analyses and D. Lang for help in the laboratory. Financial support from the Arnold Arboretum of Harvard University is gratefully acknowledged.

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Corresponding Editor: G. Orti