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MOLECULAR  
PHYLOGENETICS  
OF THE MAGNOLIIDAE:  
CLADISTIC ANALYSES OF  
NUCLEOTIDE SEQUENCES OF  
THE PLASTID GENE *rbcl*<sup>1</sup>

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ABSTRACT

Nucleotide sequences of the plastid protein-coding gene *rbcl* from 64 species of 36 families in subclass Magnoliidae sensu Cronquist and representatives of all other major seed plant groups were analyzed by parsimony in a series of four analyses. *Ceratophyllum* (Ceratophyllaceae) was found to be sister to all other angiosperms. Other magnoliids formed five major groups, roughly corresponding to the Magnoliales, Laurales, Aristolochiaceae/Piperales, Nymphaeales, and Ranunculales/Papaverales. Four magnoliid lineages, those with monosulcate or monosulcate-derived pollen (Magnoliales, Laurales, Aristolochiales/Piperales, and Nymphaeales), and the monocots (with the same type of pollen) formed a weakly supported monophyletic group. The Illiciales (Illiciaceae and Schisandraceae), with a unique type of tricolpate pollen, also fell into this monosulcate clade. Relationships among these major lineages were resolved in all trees, but with low levels of support. Magnoliidae with tricolpate and tricolpate-derived pollen, Ranunculales/Papaverales and Nelumbonaceae, fell into the other large clade of angiosperms, which have sometimes been termed the eudicots. Monophyly of this large tricolpate clade was strongly supported. These results demonstrated a high degree of congruence with the major groups identified in other studies, but they differed in the general relationships of these groups, particularly the monophyly of the monosulcate taxa and the sister-group relationship of angiosperms with the two general pollen types. They also deviated in the specific taxon composition of some major clades.

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The dicotyledonous subclass Magnoliidae (Cronquist, 1981) contains most of the putatively primitive angiosperms. A thorough phylogenetic understanding of the complex is critical for study of the origin and reconstruction of the phylogeny of angiosperms. The Euanthial Theory of angiosperm evolution (Arber & Parkin, 1907) postulates that angiosperms with large strobiloid flowers such as Magnoliales are more primitive than those with diminutive, simple flowers (e.g., Piperales and Chloranthaceae). Over the past two decades, however, paleobotanists have accumulated a large body of

fossil evidence which suggests that, during their early evolution, angiosperms with small and simple flowers might have paralleled or preceded those with large and complex flowers (Dilcher, 1979; Upchurch, 1984; Crane et al., 1986; Friis et al., 1986; Crane, 1989; Taylor & Hickey, 1990; Pedersen et al., 1991). A similar challenge has come from studies of extant plants (Burger, 1977, 1981; Hamby & Zimmer, 1992; Taylor & Hickey, 1992). Furthermore, which particular groups of Magnoliidae are related to the monocots and eudicots (the dicots with tricolpate and tricolpate-derived pollen

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types, exclusive of Illiciales, cf. Walker & Doyle, 1975; Doyle & Hotton, 1991) remains a debatable issue (Dahlgren & Clifford, 1982; Dahlgren et al., 1985; Walker & Walker, 1984; Donoghue & Doyle, 1989; Doyle & Hotton, 1991).

The Magnoliidae have been known as the ranalian complex, Ranales, or Polycarpicae. In the classification scheme of A. P. de Candolle (1824), their prototype first appeared; it included key families of Magnoliales (Magnoliaceae, with *Illicium* of Illiciaceae and *Drimys* and *Tasmannia* of Winteraceae; and Annonaceae, with *Kadsura* of Schisandraceae), Nymphaeales (Nymphaeaceae, with *Nelumbo* of Nelumbonaceae), and Ranunculales/Papaverales (Ranunculaceae; Menispermaceae, with *Lardizabala* of Lardizabalaceae; Berberidaceae; Papaveraceae; and Fumariaceae). These taxa were placed sequentially at the beginning of the system, which covered only dicots and gymnosperms, but no collective name was given to this aggregation of families. Lindley (1833) was the first one to erect two taxa, Ranales and Anonales, to cover this group of plants. In *Genera Plantarum*, Bentham & Hooker (1862) followed de Candolle's treatment for this complex, except for addition of a lauralean family, the Calycanthaceae; they placed all these taxa together under Ranales. Situating these families at the beginning of the classification systems antedated formulation of the Euanthial Theory by Arber & Parkin (1907), which equated flowers of the Magnoliales to Bennettitalean strobili and provided a theoretical explanation for recognizing Magnoliidae as the primitive angiosperms. Eichler (1890) and Engler & Prantl (1891), despite advocating Amentiferae as the primitive angiosperms, also made a significant contribution to development of the ranalian concept by adding to the complex the major lauralean families, Hernandiaceae, Lauraceae, and Monimiaceae, and several other taxa such as Lactoridaceae and Trochodendraceae (also including *Cercidiphyllum* of Cercidiphyllaceae and *Euptelea* of Eupteleaceae). In his 1915 paper, often regarded as the first truly phylogenetic classification, Bessey brought to the ranalian complex the piperalean families (Piperaceae, Saururaceae, and Chloranthaceae). By early this century, the Magnoliidae were conceptually mature and comprised five major lineages: Magnoliales, Laurales, Piperales, Nymphaeales, and Ranunculales/Papaverales.

Modern systematists (Takhtajan, 1987; Cronquist, 1988; Dahlgren, 1989; Thorne, 1992) have agreed upon the basal position of Magnoliidae in angiosperms and recognition of the five major lin-

eages; nonetheless, their opinions differ widely on relationships among these lineages. Takhtajan (1987) divided the complex into two subclasses, placing monosulcate lineages in Magnoliidae and tricolpate lineages in Ranunculidae (but Illiciales and Nelumbonaceae were in the former). Dahlgren's (1989) treatment of the complex resembled that of Takhtajan (1987), but no higher categories were given to the two assemblages, Magnolianaes/Nymphaeanaes and Ranunculanaes (cf. fig. 1 in Dahlgren, 1989). In the monosulcate assemblage, Dahlgren placed Piperales with Nymphaeales, rather than with Magnoliales and Laurales as in Takhtajan's (1987) scheme. A major difference between Cronquist's (1981, 1988) and Takhtajan's (1980, 1987) systems is that Cronquist did not recognize Ranunculales/Papaverales as a separate subclass. Thorne (1992) divided the complex into three superorders, Annonanae (including most monosulcate families plus Ranunculales/Papaverales), Nymphaeanae, and Rafflesianaes (the latter two narrowly defined).

Relationships of several isolated families, Austrobaileyaceae, Ceratophyllaceae, Chloranthaceae, Illiciaceae/Schisandraceae, Lactoridaceae, and Nelumbonaceae, have been extensively debated. These taxa are either included in one of the major lineages of Magnoliidae or treated as independent minor groups, but their affinities are typically stated to be uncertain.

Recently, several cladistic studies of morphological, phytochemical, and molecular data have attempted to answer some of the questions concerning the phylogenetics of Magnoliidae (Dahlgren & Bremer, 1985; Donoghue & Doyle, 1989; Lconte & Stevenson, 1991; Martin & Dowd, 1991; Hamby & Zimmer, 1992; Taylor & Hickey, 1992). These efforts have met with only partial success due to: (i) widespread homoplasy in primitive angiosperms, (ii) problems of choosing appropriate ingroups and outgroups, (iii) difficulty of coding characters in morphological cladistic studies, or (iv) insufficient sampling (a major problem in the molecular investigations).

Protein-coding genes have been shown to reflect the genealogical history of organisms (Atchley & Fitch, 1991), though to what degree and at what taxonomic level this is true awaits further study. Evolutionarily conservative genes, such as *rbcL*, which codes for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), are considered suitable for study of relationships among taxa of higher rank (Ritland & Clegg, 1987; Zurawski & Clegg, 1987; Palmer et al., 1988). They provide new characters that permit an in-

dependent evaluation of phylogenetic relationships among plant groups uninfluenced by previous ideas of transformation of morphological characters and putative relationships among taxa. In the present study, we perform parsimony analyses of *rbcL* sequences from species representing almost all magnoliid families and endeavor to (1) evaluate the concepts of Magnoliidae, (2) identify major lineages in the subclass, investigate relationships within these major lineages, and assess affinities of several problematic taxa, (3) determine the relative phylogenetic positions of the major lineages within Magnoliidae, and (4) examine relationships of the Magnoliidae to monocots and eudicots.

#### MATERIAL AND METHODS

Well aware of the pitfalls caused by inadequate sampling of sequence variation (Albert et al., 1993; Chase et al., 1993), we sampled at least one species from each family (this of course does not address adequately the putative monophyly of these families). Thus 64 species from 36 families representing all orders of the Magnoliidae were included (we follow the classification scheme of Cronquist, 1981, unless otherwise indicated). These taxa are listed in the Appendix at the end of this issue. Several sequences have been published previously (Golenberg et al., 1990; Les et al., 1991). Missing families are Circaeasteraceae, Gomortegaceae, Trimeniaceae, Hydnoraceae, and Rafflesiaceae (Takh-tajan, 1987; Dahlgren, 1989; Thorne, 1992). It is likely that the last two, which are achlorophyllous, lack *rbcL*, as was found in other parasitic plants (dePamphilis & Palmer, 1990).

Total cellular DNA was extracted using the modified CTAB method (Doyle & Doyle, 1987) from 0.6–2.0 g fresh or silica gel-dried leaves (Chase & Hills, 1991) collected from a single plant (in the case of *Hernandia ovigera* L., the leaf was removed from a 22-year-old herbarium specimen). All DNAs were purified by CsCl-ethidium bromide gradient centrifugation. A fragment containing the gene *rbcL* was amplified using a thermo-stable DNA polymerase and two synthetic 26-mer primers based on the *rbcL* sequences from *Zea mays* L. and *Nicotiana tabacum* L. The amplified fragment was ligated into Bluescript vector and cloned with standard recombinant techniques. Sequencing was performed using Sequenase 2.0 (US Biochemical, Inc.) for dideoxynucleotide chain-termination reactions, and sequences were obtained from both strands. For several species, the gene was directly sequenced from the amplified fragment without cloning.

Sequence data were analyzed using PAUP 3.0s (Swofford, 1991). Several searches were conducted to approach problems at different scales and with different techniques.

**Search A.** All 65 magnoliid sequences were analyzed together with 434 other seed plant *rbcL* sequences (see Chase et al., 1993) to identify major lineages in the Magnoliidae, determine relative position of these lineages, and examine relationships of the subclass to monocots and eudicots. Two sequences of *Canella winterana* (L.) Gaertner, both with intact reading frames, were obtained from the DNA sample extracted from a single plant. They differ by 57 base pairs; 24, 15, and 18 of these differences occur at the first-, second-, and third-codon position, respectively. Both sequences were used in all analyses; one of them may be a “pseudogene.” Due to the magnitude of the data set and the limitation of the software, the level of parsimony of this broad search was not certain (see Chase et al., 1993, for a more detailed explanation and discussion of the result of this larger analysis). This search helped us select a smaller number of taxa for a “manageable” analysis to examine internal support.

**Search B.** We performed another analysis with all monosulcate magnoliid sequences plus selected gymnosperms, monocots, and eudicots that served as place-holders for their respective groups identified in Search A. An analysis under the Fitch criterion (equal weights for all substitutions; Fitch, 1971) using 2,000 random sequence additions, STEEPEST DESCENT, MULPARS (but permitting only 10 trees to be held at each step), and NNI (nearest-neighbor interchange) found only one island of trees. All trees within this island were recovered by using the trees found in the random searches as starting points with MULPARS and TBR (tree bisection-reconnection) until swapping was completed.

**Search C.** To examine the effect of distant outgroups such as Gnetales on the ingroup network, we conducted an ingroup-only analysis of flowering plants. Again, this analysis was performed under the Fitch criterion using 2,000 random sequence additions in the same manner as described above. In addition to the same set of monosulcate magnoliids and monocots as were used in Search B, representative species of all families in the Ranunculales/Papaverales were included to investigate interfamilial relationships within this group. Two islands of equally parsimonious trees were found, and all of the trees in each of those islands were recovered by the same method as in Search B (swapping to completion with MULPARS and TBR).

To gain a measure of the robustness of the topology, decay of parsimony (Bremer, 1988) was examined. All maximum parsimony trees of both islands were used as starting trees to search for trees up to five steps less parsimonious; the FILTER TREES option was used to identify trees at each length, and a strict consensus tree was computed at each step. The number of steps less parsimonious at which a branch collapsed was recorded as the decay index. The larger the number, the more robust a branch. A decay index of 1 indicates that the branch is present in all maximum parsimony trees but collapses at maximum parsimony plus one step.

**Search D.** There has been great interest in recent cladistic studies to determine the basalmost lineage of angiosperms. The Nymphaeales are often claimed as a sister group to all other angiosperms (Donoghue & Doyle, 1989; Hamby & Zimmer, 1992), but our results did not place them in such a position. To evaluate the hypothesis of Nymphaeales being the basalmost angiosperms, we performed a topology constraint experiment using the same set of taxa as in Search B, but with the Nymphaeales constrained to be sister to all other angiosperms (using the CONSTRAINTS option of PAUP). The search was conducted under the Fitch criterion using 2,000 random sequence additions in the same manner as described in Search B. This is a more accurate means to determine the loss of parsimony associated with an alternate topology; merely constraining the topology based on the shortest trees found overall without performing branch swapping is likely to result in an inaccurate assessment of tree length because it does not permit character transformations to be optimized on different branches than in the unconstrained trees.

## RESULTS

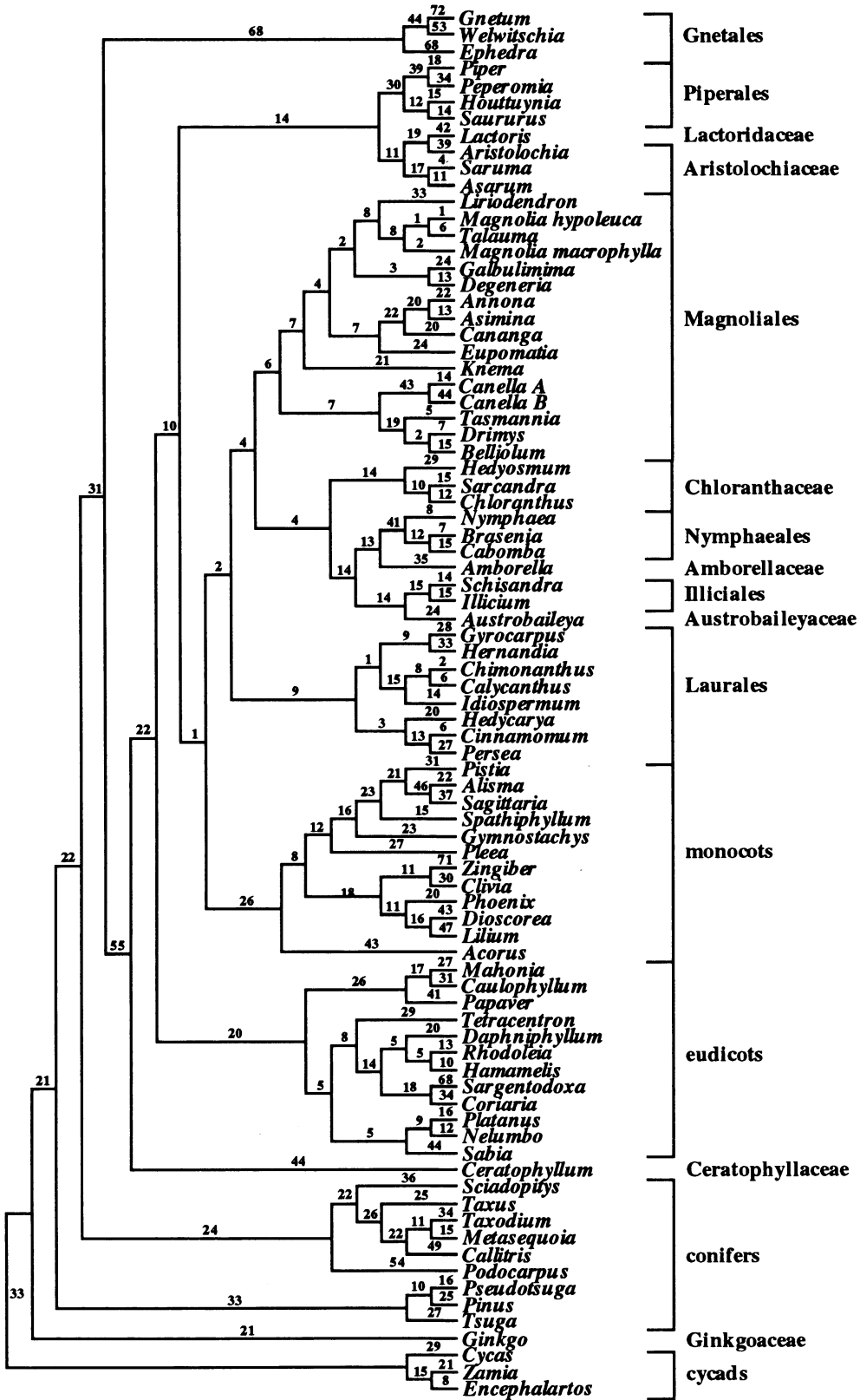
**Search A.** A strict consensus tree was computed from 3,900 equally parsimonious trees at a length of 16,305 steps (Chase et al., 1993). These trees have a consistency index (C.I.; for potentially synapomorphic characters) of 0.102 and a retention index (R.I.) of 0.638; a summary of this tree is shown in Figure 1 (for the detailed version of the strict consensus tree, see Chase et al., 1993). Exclusive of *Ceratophyllum*, which is sister to all other flowering plants, the angiosperms are split into two clades that correspond to the two general pollen types, monosulcate and tricolpate (for the sake of convenience, we refer to angiosperms with monosulcate and monosulcate-derived pollen types

as the "monosulcates" and those with tricolpate and tricolpate-derived pollen types as the "eudicots," the latter in accord with Doyle & Hotton's, 1991, use of that term). Five major lineages are identified among the Magnoliidae; they are roughly equal to the Magnoliales, Laurales, Piperales (including Aristolochiaceae and Lactoridaceae), Nymphaeales (excluding Nelumbonaceae but including Amborellaceae, Austrobaileyaceae, Chloranthaceae, and Illiciales), and Ranunculales (excluding Coriariaceae, Sargentodoxaceae, and Sabiaceae but including Eupteleaceae, Fumariaceae, and Papaveraceae). (We will use these ordinal circumscriptions throughout the rest of this paper; i.e., under Piperales we will include Aristolochiaceae and Lactoridaceae, unless stated otherwise.) The four magnoliid lineages with monosulcate pollen types, (i) Magnoliales, (ii) Laurales, (iii) Piperales, and (iv) Nymphaeales, plus the monocots (with the same type of pollen), form a clade. The Illiciales, which have anomalous tricolpate pollen (see below), also fall into this monosulcate clade. The Piperales are sister to all other monosulcates; Magnoliales/Nymphaeales, Laurales, and monocots form an unresolved trichotomy in the strict consensus tree. The Ranunculales, Nelumbonaceae, and Sabiaceae, all of which have tricolpate pollen, together with other angiosperms possessing the same pollen type, form a monophyletic group. This large clade corresponds to Walker & Doyle's (1975) nonmagnoliid dicots or Doyle & Hotton's (1991) "eudicots." The Ranunculales are sister to the rest of the eudicots. In another basal lineage of eudicots are Nelumbonaceae and Sabiaceae (with Platanaceae and Proteaceae; hamamelid I, Fig. 1). The Coriariaceae and Sargentodoxaceae are not affiliated with the Magnoliidae, the former being sister to the clade of Cucurbitaceae/Begoniaceae/Datisceae and the latter imbedded in the Fabaceae (both in rosid I, Fig. 1; also see fig. 11B in Chase et al., 1993).

**Search B.** A single island of 46 trees at a length of 3,414 steps with C.I. of 0.275 (unique substitutions excluded) and R.I. of 0.566 was found. One of these maximum parsimony trees favored by the weighting criterion (Albert et al., 1993) is shown with Fitch branch lengths optimized on it (ACCTRAN optimization; Fig. 2). Even though far fewer taxa (82 species) were used in Search B, the same general topology as Search A was obtained.

**Search C.** Two islands of 89 and 3 trees respectively, at a length of 2,674 steps with C.I. of 0.346 (unique substitutions excluded) and R.I. of 0.531 were found. For illustration, one tree from each island was selected using the weighting criterion of Albert et al. (1993). The trees in the





larger island (Fig. 3), though less resolved, are congruent in the general topology with the trees from Search B (Fig. 2). In contrast, the trees of the smaller island (Fig. 4) are different from those found in Search B; *Ceratophyllum* allies with *Canella* in the Magnoliales rather than being situated in an isolated position. These trees were arranged to agree with the results of two previous searches (i.e., with the eudicots sister to the monosulcates).

Many parts of this topology (Fig. 3) do not demonstrate strong internal support (decaying at two or fewer steps less parsimonious). At the level of the relationships among the major groups identified, all are weakly supported (even though the monophyly of some of the orders themselves are strongly supported; see below). The shift of *Ceratophyllum*, which is the only taxon with a different position in the two islands of trees, demonstrates its low level of internal support. It would appear that the use of distant outgroups such as Gnetales may be introducing homoplasy such that the smaller island does not exist at maximum parsimony in Search B (we cannot evaluate the degree of parsimony of the trees or the existence of other islands of equally parsimonious trees in Search A because of the size of the data set). Groups with moderate to strong support (decaying at three or more steps less parsimonious; Fig. 3) are eudicots, monocots, Laurales, "core" Magnoliales (excluding Canellaceae and Winteraceae), Nymphaeales (including the associated woody families, but excluding Chloranthaceae), Piperales, and Ranunculales.

**Search D.** A single island of 186 trees at a length of 3,425 steps with C.I. of 0.274 (unique substitutions excluded) and R.I. of 0.565 was found. One of the maximum parsimony trees chosen by the weighting criterion is shown in Figure 5. Compared with the trees from Search B (length of 3,414 steps; the same taxa are present in both), the trees recovered in this search are only slightly less parsimonious: 11 steps or only 0.32% longer.

Although relationships among the four monosulcate magnoliid lineages, monocots, and eudicots are resolved with only low levels of support, these clades are consistently identified, and relationships within the clades remain unchanged in the series of analyses (Figs. 1, 2, 3, 4). Even though the constraint experiment makes the group related to the Nymphaeales paraphyletic to all other angio-

sperms, it leaves them in near proximity to each other and otherwise identifies the same major lineages as in the previous searches. *Ceratophyllum* is the single taxon that shifts wildly with each treatment.

## DISCUSSION

### 1. PHYLOGENETIC EVALUATION OF THE MAGNOLIIDAE

From the review of historical development of the ranalian concept and the treatment of the complex in several modern classification systems presented in the introduction, it is clear that five major lineages, Magnoliales, Laurales, Piperales, Nymphaeales, and Ranunculales, were consistently recognized. In this study, we obtained clades that are roughly equal to these traditionally recognized lineages, and they occupy basal positions among angiosperms. Our analyses were conducted only under the criterion of parsimony, and we did not rely on any previous hypothesis concerning evolution of morphological characters or putative relationships among these taxa. The high degree of correspondence between the clades identified in this study and those traditionally recognized categories provides support for these lineages (even though support for several of these was weak in our study) and is an indication that *both* morphology and *rbcL* sequences are informative for phylogenetic reconstruction.

The split of the angiosperms, exclusive of *Ceratophyllum*, into a monosulcate and eudicot clade in our results was unexpected. Monophyly of the monosulcates has never been suggested, and at present we know of no unequivocal morphological or phytochemical characters to define the clade. Possession of monosulcate pollen is not a synapomorphy because this pollen type is also found in nonflowering seed plants. The ethereal oil cells could be a potential synapomorphy for the monosulcates; they occur in all component lineages (at least in some basal members) of this clade and absence of these cells in Amborellaceae/Nymphaeales and most monocots (basal monocots such as *Acorus* have the ethereal oil cells, Cronquist, 1981) can be interpreted as due to secondary loss. A close relationship between the Magnoliidae and the monocots has been proposed by Huber (1977), Hegnauer (1977), and Kubitzki & Gottlieb (1984),

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FIGURE 2. One of the 46 maximum-parsimony (Fitch) trees from the single island found for *rbcL* sequences of 82 species of gymnosperms, monosulcate magnoliids, monocots, and eudicots. This particular tree was selected by the weighting criterion of Albert et al. (1993), but branch lengths are Fitch optimizations (ACCTRAN).

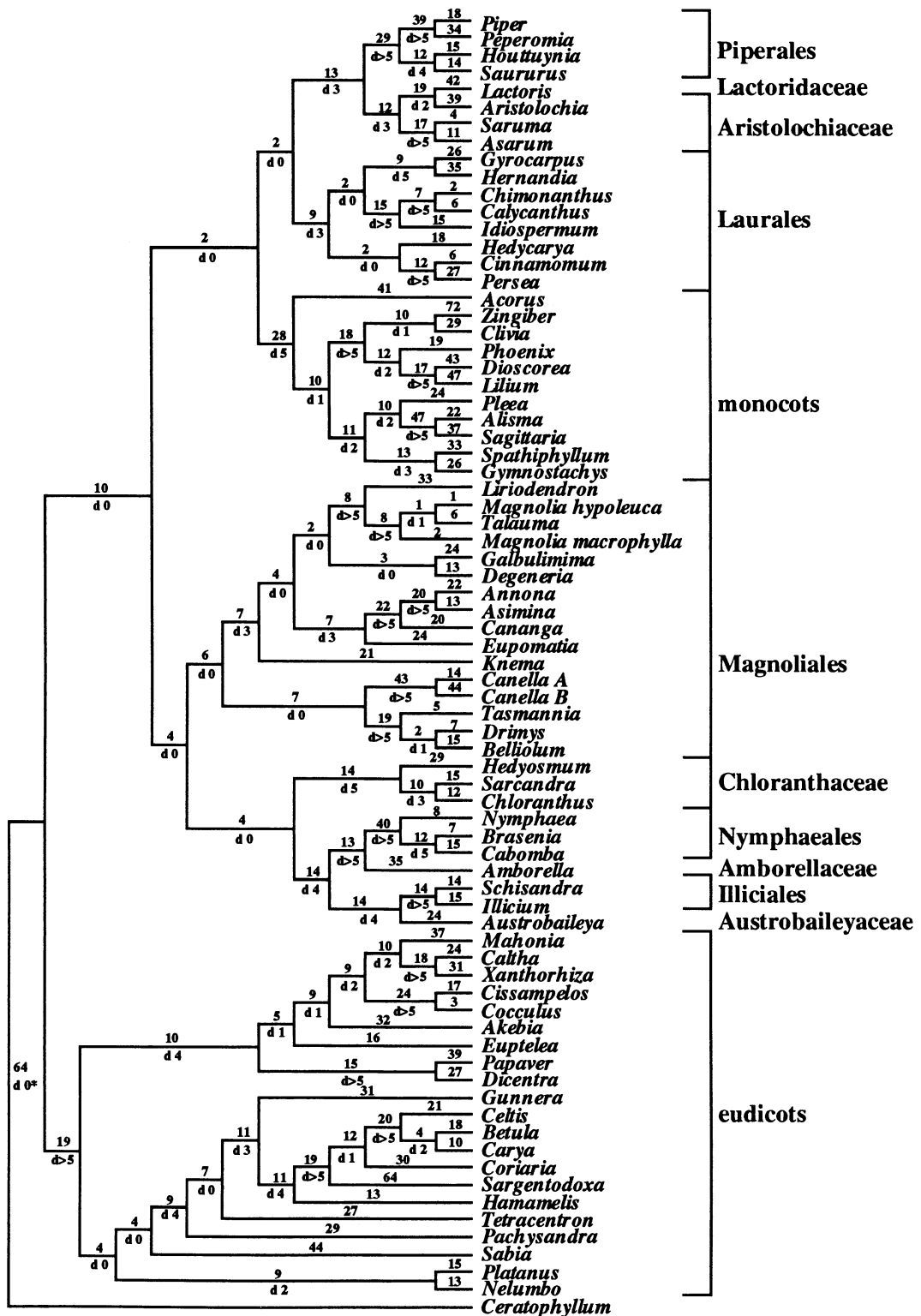


FIGURE 3. One of the 89 maximum-parsimony (Fitch) trees, chosen with the weighting criterion, from the larger of the two islands found for *rbcL* sequences of 74 species of angiosperms. The branch lengths are Fitch optimizations (ACCTRAN). Decay indices are indicated below each branch and are preceded with a "d" ("d0" indicates a branch present in the particular tree favored by the weighting criterion but not in all maximum-parsimony trees).



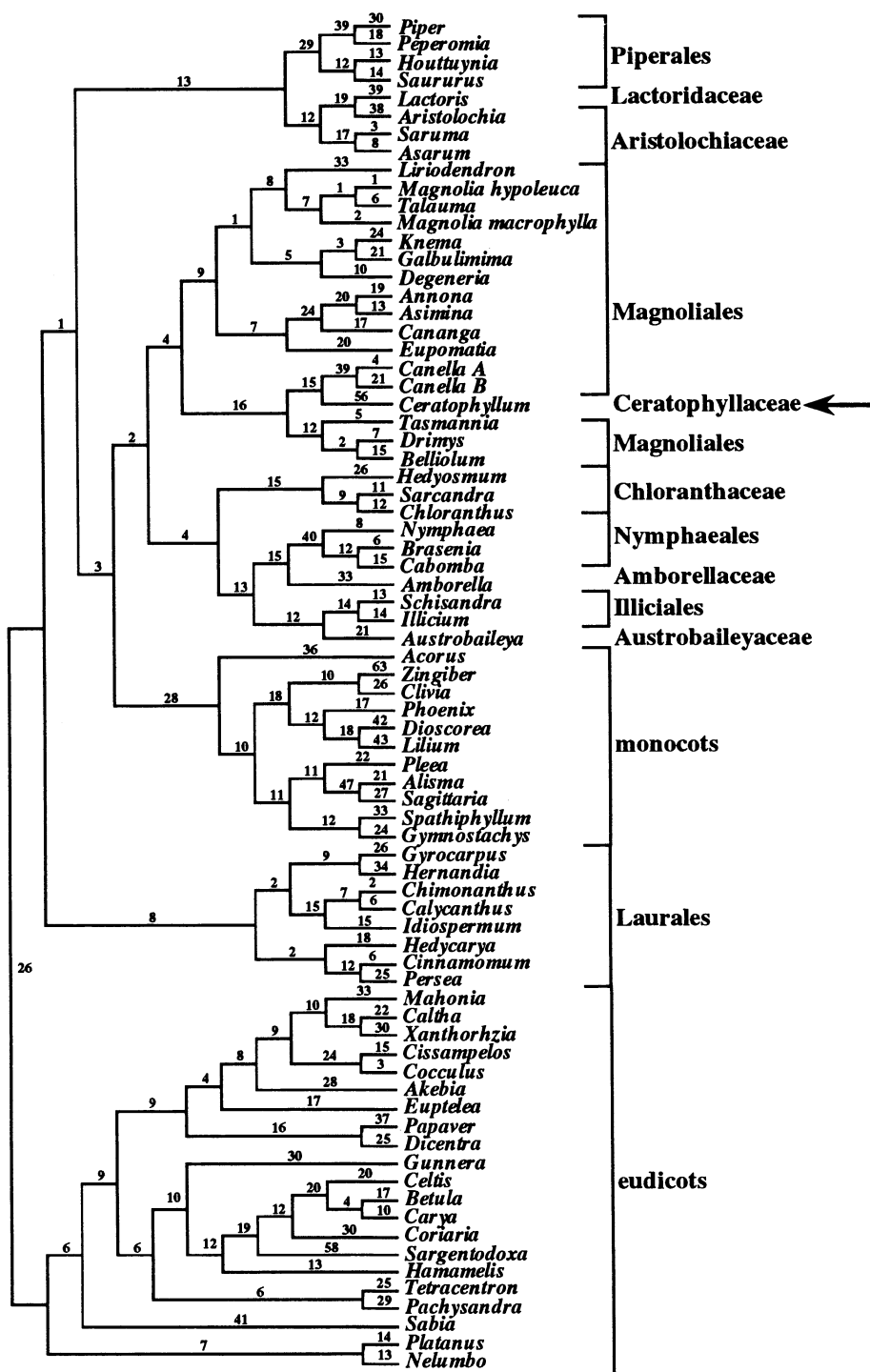
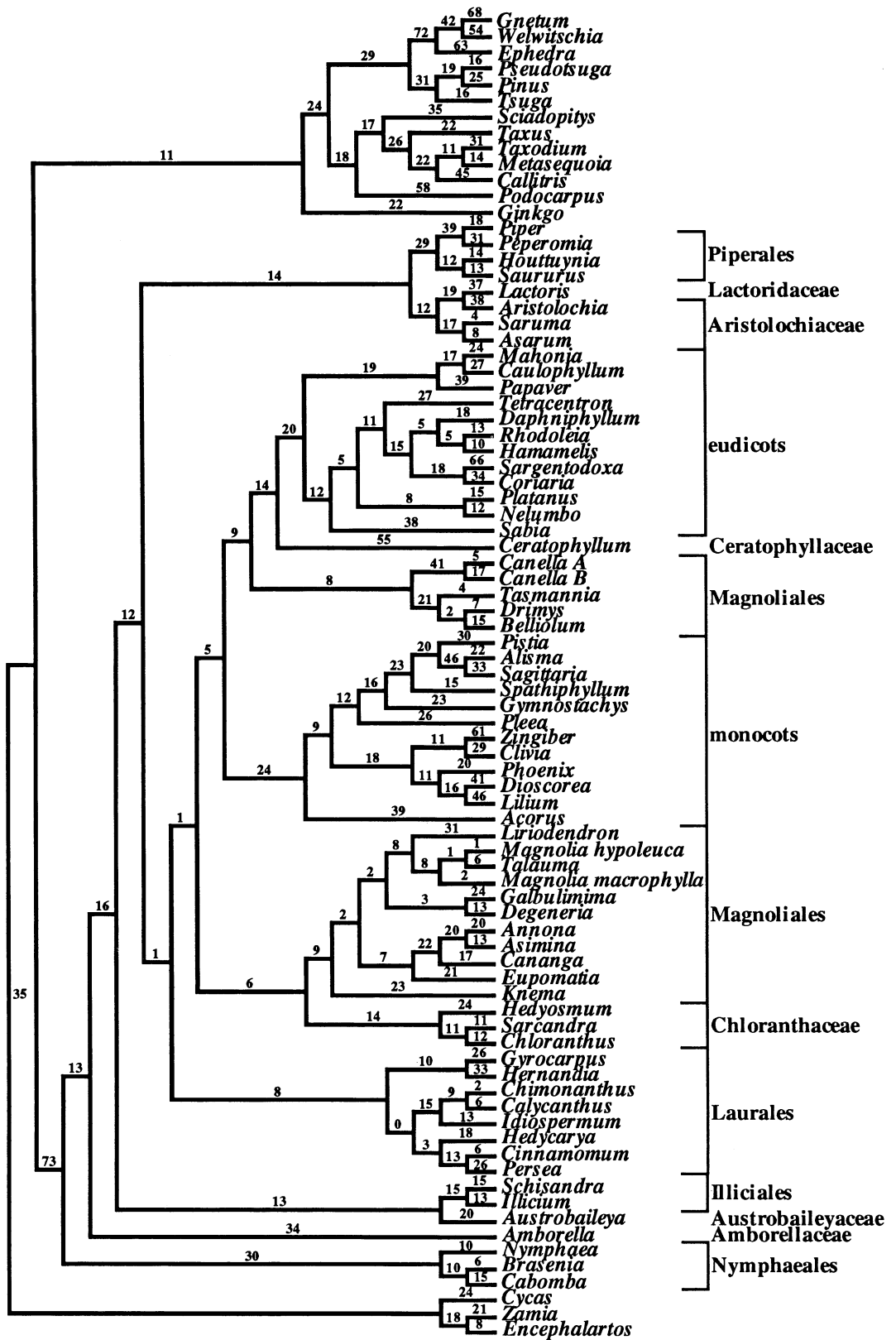


FIGURE 4. One of the three maximum-parsimony (Fitch) trees, chosen with the weighting criterion, from the smaller of the two islands found for *rbcL* sequences of 74 species of angiosperms. The branch lengths are Fitch optimizations (ACCTRAN).



but they relied on what are probably symplesiomorphic features. Currently available fossil evidence indicates that monosulcate angiosperms antedate eudicots (Doyle, 1969; Muller, 1970, 1981, 1984; Doyle & Hickey, 1976; Hickey & Doyle, 1977; Friis & Crepet, 1987; Hughes & McDougall, 1990; Doyle & Hotton, 1991), and it has been postulated that the latter were derived from the former (Walker & Walker, 1984). Crane & Lidgard (1990), after examining palynological diversity in the Cretaceous, suggested that the monosulcates probably represent an evolutionary grade rather than a clade. Three different scenarios may explain the discrepancy between our results and these previous hypotheses. First, the fossil evidence is most certainly incomplete, and future paleobotanical exploration may find older evidence for eudicots. Second, the weakly supported monosulcate clade is perhaps generated spuriously by high levels of sequence divergence among the monosulcates, eudicots, and gymnosperms (i.e., the monophyletic monosulcate clade is the result of "long branch attraction"; cf. Felsenstein, 1978). The third explanation postulates that two lines, both of which contained monosulcates, existed: one was paraphyletic to extant eudicots, and the monosulcate members of this line are now extinct; the other line included at the least all extant monosulcates. This third scenario implies the existence of yet unidentified lineages of monosulcates in the fossil record.

It has long been recognized by phylogenists that Magnoliidae sensu Cronquist are paraphyletic to monocots and eudicots. A cladistic study of basal angiosperms using morphological characters has corroborated this viewpoint (Donoghue & Doyle, 1989). Our study of *rbcL* sequences further demonstrates the paraphyly of the Magnoliidae. Takhtajan (1987) and Dahlgren (1989) removed the Ranunculales from the Magnoliidae and placed them in a higher category equal to the rest of the subclass. These plants lack several features used to define the Magnoliidae, e.g., spherical ethereal oil cells in the parenchymatous tissues and monosulcate pollen. In our analyses, the Ranunculales consistently fall into the strongly supported eudicot clade apart from the rest of the Magnoliidae sensu Cronquist.

The Nymphaeales are another member of the

Magnoliidae that are often treated as an independent group of equal rank to the rest of the subclass (Thorne, 1992) or to all other monosulcate members of the subclass (i.e., excluding Ranunculales; Walker & Walker, 1984; Takhtajan, 1987). This study does not support such treatment. The Nymphaeales are only one of the major lineages in monosulcate Magnoliidae, and furthermore, several other taxa, Amborellaceae, Austrobaileyaceae, and Illiciales, share a close, unique phylogenetic relationship with them.

The enigmatic genus *Ceratophyllum* forms its own clade and is sister to all other angiosperms when outgroups to the angiosperms are included, but when only angiosperms are analyzed its highly divergent sequence is involved in two islands of equally parsimonious trees in which it occurs in radically different positions (Figs. 3, 4). The affinities of *Ceratophyllum* have been controversial, and most recent authors favored a relationship with *Cabomba* of the Cabombaceae (Cronquist, 1981; Takhtajan, 1980; Dahlgren, 1989). After a critical review of all the evidence, Les (1988) questioned this connection. The phylogenetic position of *Ceratophyllum* shown in this study largely corroborates Les's (1988; Les et al., 1991) proposition that the genus represents a vestige of the ancient angiosperms that diverged early from the line leading to most other modern taxa. From Aptian sediments in Victoria, Australia, and Albian and Cenomanian sediments in Kansas, North America, Dilcher (1989) reported fossil fruits similar to those of extant *Ceratophyllum* species; fossil leaves resembling those of modern *Ceratophyllum* were also found associated with the fruits. Possession of a suite of distinctive characters (partially sealed carpels, unitegmic ovules, branching pollen tubes, and lack of exine, vessels, and perianth) makes the genus anomalous among angiosperms.

No evidence, however, can be considered to give strong support for any of the placements of *Ceratophyllum* in our *rbcL* trees. The inclusion of distantly related outgroups (in particular, the highly divergent Gnetales) may be responsible for the placement of *Ceratophyllum* as sister to the rest of the angiosperms by introducing spurious synapomorphies. Nevertheless, in the search with non-flowering seed plants removed (Search C), *Cera-*

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FIGURE 5. One of the 186 maximum-parsimony (Fitch) trees found in a constraint experiment in which the Nymphaeales were specified as the sister of all other angiosperms. These trees are only 11 steps less parsimonious than the most parsimonious, unconstrained trees found with the same data matrix. The branch lengths are Fitch optimizations (ACCTRAN).

*tophyllum* remains as an outlier to the monosulcates and eudicots in the trees of one island (Fig. 3). In the trees from the other island, the genus is allied with *Canella* in the Magnoliales (Fig. 4), which seems unsatisfactory, both from the perspective of the long branches of *Ceratophyllum* and *Canella* and lack of corroboration from other evidence. In the topology constraint experiment (Search D), *Ceratophyllum* again ends up in another position that is potentially spurious (Fig. 5). Our overall conclusion is that the *rbcL* analyses do not definitively resolve the phylogenetic relationships of *Ceratophyllum*, but they do suggest, in agreement with Les's hypothesis (1988, 1991), that it is distantly related to all other extant angiosperms and may represent an ancient and highly modified taxon allied to the oldest angiosperms, which is consistent with its long fossil history.

## 2. PHYLOGENETICS OF THE MAGNOLIALES, LAURALES, PIPERALES, NYMPHAEALES, AND RANUNCULALES

The isolated position of Winteraceae has been recognized for some time (Bailey & Nast, 1943; Ehrendorfer et al., 1968; Thorne, 1968; Walker, 1976a; Takhtajan, 1980), and the family is placed independently in several classification systems (Walker & Walker, 1984; Takhtajan, 1987; Dahlgren, 1989; Thorne, 1992). A morphological cladistic study showed that Winteraceae fall outside of the "core" Magnoliales (Donoghue & Doyle, 1989). The family has several features that are not compatible with other members of Magnoliales, e.g., vesselless wood, poorly organized leaf venation, and ulcerate pollen shed in permanent tetrads. The Canellaceae are another family not securely placed in the Magnoliales (Donoghue & Doyle, 1989). A relationship between these two families emerges from this study as well as previous work. Leroy (1977) suggested that Winteraceae are allied to Canellaceae via the anomalous Madagascan genus *Takhtajania*; both have a compound unilocular ovary, a type of gynoecium rare in the Magnoliales. The two families (plus the Illiciales) were found to be associated in some of the equally most parsimonious trees in Donoghue & Doyle's (1989) study. Gottlieb et al. (1989) found that among members of Magnoliales and Piperales sensu Dahlgren, Winteraceae and Canellaceae share sesquiterpenoids of the drimane and rearranged drimane type, and at least two representatives of the drimane type occur only in these two families. A recent study of 26S rDNA also showed that these two families are related (Suh et al., 1992).

The other families of the Magnoliales, Anno-

naceae, Degeneriaceae, Eupomatiaceae, Himantandraceae, Magnoliaceae, and Myristicaceae, are often recognized as "core" Magnoliales (Thorne, 1976; Walker, 1976a). Donoghue & Doyle's (1989) morphological cladistic study also identified this group. Our study of *rbcL* sequences offers moderate support for this group (Fig. 3). The Myristicaceae, traditionally regarded as an advanced member of Magnoliales (Walker, 1976b; Walker & Walker, 1984), occupy a weakly supported basal position in this clade (Fig. 3).

The anomalous magnoliaceous genus *Liriodendron* stands in an isolated position from the rest of the family, and its inclusion in the Magnoliaceae has been questioned by Barkley (1975). The relationship of *Liriodendron* to other magnoliaceous members is well supported in this study (Fig. 3). A reliable morphological character supporting monophyly of Magnoliaceae is their gynoecium, spirally arranged follicles on an elongate receptacle, which elsewhere is only found in Ranunculaceae. The mid-Cretaceous (Cenomanian) angiospermous fructification *Archaeanthus* provides evidence for a long history of this type of fruit (Dilcher & Crane, 1984). Another character defining the Magnoliaceae is their large stipules that enfold terminal buds. The rest of the Magnoliaceae are so homogenous that their taxonomy is notoriously difficult (Dandy, 1964; Law, 1984; Nooteboom, 1985), and *rbcL* sequences show little divergence among the genera (Qiu, Chase & Parks, unpublished).

In Laurales, *Hernandia* and *Gyrocarpus* have been placed in two unrelated families, Hernandiaceae and Gyrocarpaceae, by some (Walker & Walker, 1984; Takhtajan, 1987). Our study shows that the relationship between these two genera is strong even though their *rbcL* sequences are divergent (Fig. 3). Dahlgren's (1983, 1989) inclusion of these two genera in the Lauraceae is not supported by this study.

The Calycanthaceae and Idiospermeaceae form a strongly supported clade, though resolution of their relationships to other lauralean families is poor. Interfamilial relationships within the Laurales are not well resolved (Fig. 3). Lack of several important taxa, such as *Trimenia*, *Gomortega*, *Atherosperma*, and other monimiaceous genera, may be a problem. Inclusion of these taxa in future studies should help to improve the resolution.

The relationship between Aristolochiaceae and Piperaceae/Saururaceae has not been consistently recognized. Takhtajan (1987) and Cronquist (1988) placed the two groups together, whereas Dahlgren (1989) and Thorne (1992) allied the Aristolochi-

aceae with the "core" Magnoliales rather than with the Piperales. Results from our study clearly favor association of the two groups.

The affinity of Lactoridaceae is an extensively debated issue. In the past, this family has been placed in the Magnoliales (Cronquist, 1981), Laurales (Takhtajan, 1980), Piperales (Walker & Walker, 1984; Thorne, 1992), or in its own order Lactoridales between the Magnoliales and Winterales (Dahlgren, 1989). A study of the family with numerical approaches suggested a magnolialean affinity (Lammers et al., 1986), but the proposition has been criticized by Carlquist (1990) on the basis of wood anatomy. Recent cladistic investigations using morphological data indicated a relationship to the Piperales (Dahlgren & Bremer, 1985; Donoghue & Doyle, 1989). A detailed comparative study of wood anatomy revealed that the Chloranthaceae, Piperaceae, and Lactoridaceae are similar in wood anatomy (Carlquist, 1990). In our analyses, support for a relationship of *Lactoris* to *Aristolochia* is weak, but alliance of the genus within Piperales is moderately supported (Fig. 3). *Aristolochia* has a rather divergent sequence, and more sampling in future studies may help to characterize better the affinities of the genus and *Lactoris*.

The Nymphaeales are distinct among primitive angiosperms in that they occupy a peculiar ecological niche and exhibit many characteristics of aquatic adaptation. How they are related to the rest of the Magnoliidae has been a long-standing issue in the phylogenetics of primitive angiosperms. In this study, we found the Nymphaeales are allied with several isolated taxa, Amborellaceae, Austrobaileyaceae, and Illiciales, whose relationships have also been widely debated. This topology is rather novel, but it is not drastically different from some previous ideas. Takhtajan (1980) and Cronquist (1981) suggested the Nymphaeales are derivatives of a woody ancestor with magnolialean features. The Austrobaileyaceae and Illiciales share many floral features with the Magnoliales and have been treated as members of that order in the past. The Amborellaceae are generally regarded as a basal member of the Laurales and have been placed near the Magnoliales (Cronquist, 1981). Hence, it is not unreasonable that these taxa form a group paraphyletic to Nymphaeales.

The Amborellaceae are a poorly studied monotypic family endemic to New Caledonia and have always been treated as a member of the Laurales. Bailey & Swamy (1948), however, found that the only species of the family, *Amborella trichopoda* Baill., lacks vessels and then questioned its placement in the Monimiaceae. Whether the angio-

sperms are primitively vesselless is an unresolved issue (Young, 1981; Donoghue & Doyle, 1989; Carlquist, 1992). It is possible that the Nymphaeales are secondarily vesselless (Cronquist, 1981). If vessellessness in primitive angiosperms is indeed due to secondary loss, this character may be of significant phylogenetic value in associating *Amborella* with the Nymphaeales. Another line of evidence supporting the relationship between *Amborella* and Nymphaeales is their lack of ethereal oil cells in parenchymatous tissues (Money et al., 1950; Cronquist, 1981). The ethereal oil cells are a characteristic feature of monosulcate Magnoliidae, and are otherwise found in all members. Finally, both Amborellaceae and Nymphaeales have granular pollen that has no or reduced endexine in the nonaperture regions of the exine, features that elsewhere are found only in the "core" Magnoliales, Trimeniaceae and some Chloranthaceae (Walker, 1976b). Although these "negative" characters need to be investigated further, they support a relationship between Nymphaeales and Amborellaceae.

The Illiciales stand as the only exception of a tricolpate pollen-bearing taxon among the monosulcate angiosperms. Previously, Walker & Doyle (1975) have highlighted their problematic aperture type. Huynh (1976, cited in Doyle & Hotton, 1991) revealed that the tricolpate condition in Illiciales is of a different type from that of eudicots; the three colpi are oriented according to Garside's rule, not Fischer's rule as in the latter. This implies that the tricolpate condition evolved independently in these two lineages. Our results support such interpretation. Leaf architecture (Hickey & Wolfe, 1975) and morphological cladistics (Donoghue & Doyle, 1989) also favor this hypothesis, though neither of these studies associates Illiciales with the particular group of monosulcate magnoliids as identified in this study. A relationship between Illiciales and Winteraceae has been proposed by some workers (Walker, 1976a; Donoghue & Doyle, 1989; Doyle et al., 1990) who relied partly on palynological evidence, yet such a relationship was not observed in investigations of chromosome number, leaf epidermis, and secondary compounds (Ehrendorfer, 1968; Baranova, 1972; Gottlieb et al., 1989). The one pollen character, coarsely reticulate and semitectate exine, that unites the two groups in Donoghue & Doyle's (1989) cladistic study has not been observed in *Walkeripollis*, the Early Cretaceous fossil pollen of putative winteraceous affinity (Doyle & Hotton, 1991).

The Austrobaileyaceae are yet another poorly studied family. They have been allied to Monimi-

aceae in Laurales (Bailey & Swamy, 1949) or Annonaceae of Magnoliales (Endress, 1980, 1983; Endress & Honegger, 1980). As noted by Cronquist (1981), the family has a combination of characters that make its placement in either order difficult: unilacunar nodes, opposite leaves, climbing habit, laminar stamens, hypogynous flowers, monosulcate pollen, and pluriovulate carpels. The relationship between Austrobaileyaceae and Illiciales identified in this study is supported by their similar sieve-element plastids (Behnke, 1988).

Given the extensive fossil record of Chloranthaceae (Couper, 1958; Kuprianova, 1967; Hughes et al., 1979; Muller, 1981; Upchurch, 1984; Walker & Walker, 1984; Friis et al., 1986; Chlonova & Surova, 1988), it may be safe to suggest that the family was more diverse in the past. A recent report of chloranthaceous fruits with anatropous ovules, instead of orthotropous ovules as in extant species, from Cenomanian sediments in eastern North America corroborates such a hypothesis (Pedersen et al., 1991). The modern family stands in a rather isolated position and has been placed in the Laurales (Thorne, 1992), Piperales (Cronquist, 1988), in its own order Chloranthales between Lactoridales and Piperales (Takhtajan, 1987), or between Winterales and Illiciales (Dahlgren, 1989). In this study, we found the Chloranthaceae are allied with Austrobaileyaceae, Illiciales, Amborellaceae, and Nymphaeales, but this position is weakly supported (Fig. 3). It has been suggested the Amborellaceae are related to the Chloranthaceae on the basis of floral morphology and anatomy (Endress, 1986a, 1987). Behnke (1988) also found Chloranthaceae share similar sieve-element plastids with Austrobaileyaceae and Illiciales.

Assignment of Ranunculales and other ranalian taxa such as Eupteleaceae, Nelumbonaceae, and Trochodendrales (Trochodendraceae and Tetracentraceae) into two subclasses, the Magnoliidae (or Ranunculidae) and Hamamelidae by Takhtajan (1987) and Cronquist (1988) may have led to a belief that these taxa are distantly related. In our analyses, they form, together with several other taxa (Buxaceae, Gunneraceae, Platanaceae, Proteaceae, and Sabiaceae), a paraphyletic group at the base of the eudicots. In comparing vegetative and reproductive morphology, Nast & Bailey (1946) dissociated *Euptelea* from *Trochodendron* and *Tetracentron*, to which they have been frequently allied (Endress, 1986b). Examinations of pollen morphology and leaf architecture corroborated the isolated position of *Euptelea* (Pragłowski, 1974; Wolfe, 1989). Nast & Bailey (1946) pointed out

that the genus is of general ranalian affinity because it possesses several features of "the syndrome of primitive characters," such as apocarpous gynoecium, incompletely sealed carpels, and numerous stamens originating in centripetal sequence. They suggested that *Euptelea* is obviously not related to the ranalian families with monosulcate pollen and ethereal oil cells, but rather to those with tricolpate pollen and no ethereal oil cells (i.e., Ranunculaceae, Berberidaceae, Lardizabalaceae, Menispermaceae, and Trochodendraceae).

The Sabiaceae were tentatively placed in the Ranunculales by Cronquist (1981). This study shows that the family is not a member of the order, but occupies a basal position in the eudicots.

The exact relationships of Nelumbonaceae have not been certain, although their affinity to the Magnoliidae is generally recognized. Tricolpate pollen, roots with vessels, and production of benzyl-isouquinoline and aporphine alkaloids are among the characters that warrant removal of this family from Nymphaeales. Serological and cytological evidence shows the family does not belong to the Nymphaeales (Simon, 1971; Raven, 1975). These characters plus absence of ethereal oil cells suggest a relationship to basal eudicots rather than the monosulcate magnoliids. Both Donoghue & Doyle's (1989) morphological cladistic study and phytochemical evidence (Gottlieb et al., 1989) support such a position.

### 3. THE BASALMOST LINEAGE OF ANGIOSPERMS

Although the Ralian Hypothesis that Magnoliidae represent the primitive angiosperms has been generally accepted, which lineage in the subclass is the most primitive remains an extensively debated issue (Friis et al., 1987). Woody Magnoliales with large strobiloid flowers and herbaceous Piperales with diminutive and simple flowers both have their proponents. Reports of chloranthoid and other non-magnolialean reproductive and vegetative structures in the Early and Middle Cretaceous (Couper, 1958; Dilcher, 1979; Krassilov et al., 1983; Upchurch, 1984; Walker & Walker, 1984; Friis et al., 1986; Crane et al., 1986; Crane, 1989; Taylor & Hickey, 1990; Pedersen et al., 1991) challenge the status of Magnoliales as the most primitive angiosperms. Cladistic studies finding that Gnetales and Bennettitales are equally closely related to angiosperms (Crane, 1985; Doyle & Donoghue, 1986) add further controversy to the debate.

Paleobotany has played an instrumental role in

elucidating the early evolution of angiosperms. A large body of fossil evidence of early angiosperms has been gathered in the last several decades, documenting presence of several major lineages of primitive angiosperms in the Early and Middle Cretaceous: Magnoliales, Laurales, Piperales, Nymphaeales, Ranunculales, and "lower" Hamamelidae (Couper, 1958; Doyle, 1969; Muller, 1970, 1981, 1984; Doyle & Hickey, 1976; Doyle et al., 1977, 1990; Hickey & Doyle, 1977; Dilcher, 1979; Hughes et al., 1979; Krassilov et al., 1983; Walker et al., 1983; Dilcher & Crane, 1984; Upchurch, 1984; Walker & Walker, 1984; Crane et al., 1986; Friis et al., 1986; Ward et al., 1989; Drinnan et al., 1990, 1991; Hughes & McDougall, 1990; Taylor & Hickey, 1990; Herendeen, 1991; Pedersen et al., 1991). These studies have greatly expanded our knowledge of early angiosperm evolution but have not answered the question of the origin of angiosperms. Further exploration of sediments of the preceding Early Cretaceous and Late Jurassic ages will be necessary before firm conclusions regarding the origin of the angiosperms are reached (Hughes & McDougall, 1987, 1990).

In a cladistic study of mostly morphological data, Donoghue & Doyle (1989) identified the Magnoliales as the basalmost lineage among angiosperms. However, they found that Nymphaeales are basal in trees only slightly less parsimonious (1 step longer or 0.56% less parsimonious; the length of their shortest trees was 178 steps). Our constraint experiment (Fig. 5) demonstrated a similar small loss of parsimony for this alternate hypothesis (0.32% less parsimonious; see Results). Hamby & Zimmer (1992) reported that Nymphaeales and Piperales are paraphyletic to other angiosperms in their investigation using ribosomal RNA sequences, but many important taxa were absent from their study. Martin & Dowd (1991) found Illiciales to be sister to all other angiosperms in their study of *rbcS* amino acid sequences. In our analyses, we found only weak support for relationships at the base of angiosperms (Fig. 3). Thus, the *rbcL* results, in spite of being resolved in favor of *Ceratophyllum* if outgroups were included, do not strongly support or refute any hypothesis of which group of Magnoliidae is sister to all other angiosperms.

#### 4. RELATIONSHIPS OF THE MAGNOLIIDAE TO MONOCOTS AND EUDICOTS

The relationship between Magnoliidae and monocots has been discussed extensively (Dahlgren & Clifford, 1982; Dahlgren et al., 1985). It is

widely believed that the two groups are closely related. Hegnauer (1977) reported that the Magnoliidae (including Ranunculales) and monocots exclusively use the tyrosine-pathway for synthesizing cyanogenic compounds, whereas other dicots exploit phenylalanine for the same purpose. In studying the distribution and biosynthesis of shikimate-derived compounds, Kubitzki & Gottlieb (1984) found that angiosperms are essentially composed of two groups, the "magnolialean block" (magnoliids, monocots, and caryophyllids) and the "rosifloreal block" (the rest of the dicots). Previously, different pairs of the magnoliids and monocots have been proposed to account for the origin of the monocots: Ranunculales/Piperales vs. Alismatales (Bessey, 1915), Nymphaeales vs. Alismatales (Takhtajan, 1969; Cronquist, 1968), Piperales vs. Arales (Burger, 1977), and Annonales vs. Dioscoreales (Dahlgren et al., 1985). Considering the heterogeneity of the Magnoliidae and a great number of similarities between the subclass and monocots (Dahlgren & Clifford, 1982; Dahlgren & Bremer, 1985), it is not surprising to see such diverse opinions about their relationship. Most, if not all, of these similarities are based on character states that are potentially symplesiomorphic or due to parallel evolution.

Although resolution of relationships at the base of angiosperms is weak in our study, several groups may be removed from the list of possible close allies to the monocots. The Ranunculales are a member of a well-supported eudicot clade and do not show any relationship to the monocots. The Nymphaeales alone are unlikely to be sister to the monocots because they show a strongly supported relationship to Amborellaceae, Austrobaileyaceae, and Illiciales. Any other major lineage of the Magnoliidae could be placed as a sister group to the monocots with only an insignificant loss of parsimony.

Monophyly and origin(s) of the eudicots have been subject to a remarkable diversity of opinions. Interpretation of traditional phylogenetic diagrams such as those of Takhtajan (1959, 1969, 1980, 1987) would lead to a conclusion that eudicots are polyphyletic and composed of several lineages independently derived from various monosulcate angiosperms with magnoliid features. Under the assumption that eudicots are derived from the monosulcates, Walker & Walker (1984) suggested that the monosulcate magnoliid families Winteraceae, Illiciaceae, Schisandraceae, and Aristolochiaceae have ties with the subclasses Ranunculidae and Caryophyllidae and that the Lactoridaceae,

Piperaceae, Saururaceae, and especially Chloranthaceae are linked to the Hamamelidae and through it to remaining dicots. In a cladistic investigation of basal angiosperms using morphological characters, Donoghue & Doyle (1989) concluded that Ranunculidae, Nelumbonaceae, and paleoherbs (including Lactoridaceae, Aristolochiaceae, Piperaceae, Saururaceae, Nymphaeaceae, Cabombaceae, and monocots) are more closely related to the Hamamelidae and other "higher" dicots than the Magnoliales and Chloranthaceae. Furthermore, they found that in some of their most parsimonious trees ranunculids and hamamelids form a monophyletic group sister to the paleoherbs, whereas in others the ranunculids are dissociated from the hamamelids and united with the paleoherbs. Doyle & Hotton (1991) reported that the oldest tricolpate pollen from the late Barremian–early Aptian of Gabon exhibits two features of typical paleoherb pollen: the sculpture consists of intermixed large and small lumina (heterobrochate) and varies from coarser at the equator to finer at the poles (graded).

The monophyly of eudicots (dicots with tricolpate and derived pollen types, including Nelumbonaceae and Takhtajan's Ranunculidae but excluding Illiciales) is strongly supported by this study. Fossil evidence from the Potomac group in eastern North America shows that all tricolpate pollen and derived types can be traced back to a morphologically generalized complex of tectate-reticulate tricolpate pollen, thus corroborating the hypothesis of single origin of the eudicots (Wolfe et al., 1975). This type of tectate-reticulate tricolpate pollen is found in the extant Ranunculales, Platanaceae, and Trochodendrales (Muller, 1984), which is consistent with placement of these taxa at the base of eudicots in the *rbcl* trees (Figs. 1–4; also see Chase et al., 1993).

Many basal eudicots are wind-pollinated, for example, Buxaceae, Eupteleaceae, Menispermaceae, Platanaceae, Tetracentraceae, and possibly Gunneraceae and Trochodendrales (Crane et al., 1991). Environmental factors such as the increasing aridity in a period immediately after the Barremian (Doyle et al., 1977; Hickey & Doyle, 1977), when tricolpate pollen first appeared (Hughes & McDougall, 1990; Doyle & Hotton, 1991), might have played a critical role in the rise of eudicots (Walker & Walker, 1984). The preponderance of wind-pollination in basal eudicots suggests that the group could be primitively wind-pollinated. Further investigation of other wind-pollinated members of the Ranunculales and "lower" Hamamelidae is needed before we have adequate information to address this question.

## CONCLUSIONS

This study of nucleotide sequences of the plastid protein-coding gene *rbcl* has identified the same major lineages as have many classification systems and previous cladistic studies of the Magnoliidae: (i) Magnoliales, (ii) Laurales, (iii) Piperales, (iv) Nymphaeales, and (v) Ranunculales. The exact familial composition of some of these clades is significantly different from any previous hypothesis. The most conspicuous difference is that Amborellaceae, Austrobaileyaceae, and Illiciales are strongly supported as members of the Nymphaeales clade. The relationship of these anomalous woody taxa to the Nymphaeales has only weak corroboration of shared absent features. Another novel feature of our results is that angiosperms fall into two monophyletic groups corresponding to the general pollen types of monosulcate and tricolpate, and they show a sister-group relationship. Our finding of the monophyletic eudicots lends strong support to pollen characters as major synapomorphies for these angiosperms. In contrast, we cannot identify any non-molecular character to define the weakly supported monosulcate clade. Resolution of relationships at the base of angiosperms is low. The position of Ceratophyllaceae as a sister group to all other angiosperms is likewise controversial, but it is supported, though not unequivocally, by the fossil evidence and the distinctive morphology of these aquatic plants.

We conclude that our molecular survey of putatively basal angiosperms gains great support from the congruence of the groups identified in this study with those recognized in classification systems and recent cladistic analyses of nonmolecular data. Our analyses of the *rbcl* sequence data were conducted merely under the criterion of parsimony, and did not rely on any previous hypothesis of evolution of morphological characters or relationships among basal angiosperms. We take this to be an indication that the molecular character systems contain historically relevant information. The general relationships among basal angiosperms, as well as composition of certain clades (e.g., Nymphaeales) identified in our *rbcl* trees, are novel and intriguing, and we suggest that they be considered as alternatives to the traditional ideas about the phylogeny of the early angiosperms. Ironically, this molecular investigation adds more controversy instead of providing solutions to the questions surrounding early angiosperm evolution. A complete understanding of the origin of angiosperms certainly requires multidisciplinary studies of both fossil and extant plants with morphological and molecular approaches.



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