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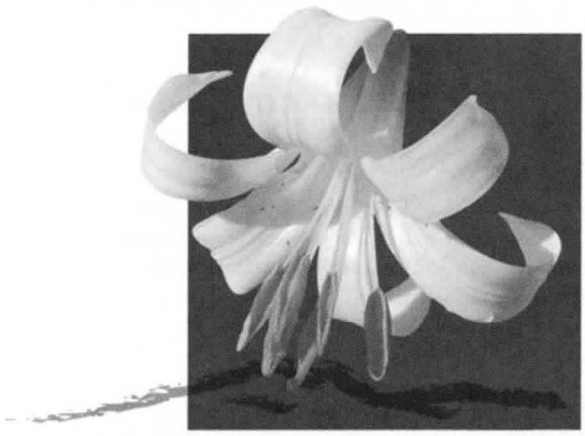

MONOCOTS

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Basal Monocots

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PLACING THE MONOCOTS: CONFLICTING SIGNAL FROM TRIGENOMIC ANALYSES

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ABSTRACT

Despite recent significant advances in understanding angiosperm phylogeny, the position of monocots remains uncertain. We present here a phylogeny inferred from four genes that unambiguously unite monocots with eumagnoliids. A well-supported position for the monocots was obtained only after we replaced the available nuclear 18S rDNA sequence data with data from phytochrome C in a matrix that also included plastid *rbc*L and *ndh*F and mitochondrial *atp*1. Over 5000 base pairs of sequence data from 42 taxa were analyzed using Bayesian inference. The results of these analyses united monocots with the eumagnoliids in a well-supported clade. Although the substitution of phytochrome C for 18S data led to a highly supported position for the monocots, comparison with more densely sampled single-gene studies revealed conflict among data sets. This indicates that larger data sets from each genome should be explored explicitly to evaluate the position of the monocots, and that each of these larger data sets also should be investigated for insight into potential sources of conflict.

Key words: Bayesian inference, eumagnoliids, monocots, *PHY*C.

INTRODUCTION

During the past decade our knowledge of angiosperm evolution has advanced substantially as a result of molecular phylogenetics. The assembly and analysis of multigene DNA sequence matrices that include hundreds of species have significantly reshaped our perception of relationships among angiosperms and the identities of the earliest lineages (e.g., Angiosperm Phylogeny Group [APG] 1998; Parkinson et al. 1999; Qiu et al. 1999; Soltis et al. 1999; Savolainen et al. 2000; Angiosperm Phylogeny Group II [APG II] 2003; and others). Paradoxically, some fundamental issues remain unresolved. Monocot placement has been referred to as "one of the next big challenges . . ." (J. D. Palmer in Palevitz 1999: 12), and this challenge remains unmet (APG II 2003).

Prior Work

Molecular phylogenetic studies that bear on the placement of monocots began with single-gene parsimony analyses of relatively few species and these expanded to studies in which hundreds of species were sampled (Chase et al. 1993; Bharathan and Zimmer 1995; Nickrent and Soltis 1995; Soltis et al. 1997). A major objective of these studies was to sample a single locus from representative species of as many recognized higher order taxa as possible. Denser taxonomic sampling was expected to break up long branches and to place poorly known or problematic species. These early studies did not resolve the placement of monocots and the monocots were non-monophyletic in some trees, notably in 18S trees (Troitsky et al. 1991; Bharathan and Zimmer 1995; Soltis et al. 1997; Duvall 2000). Single-gene and single-genome data sets were examined for evidence of phylogenetic conflict and data combination was explored (e.g., Savolainen et al. 2000). Multigene and multigenome analyses began with genes that had been most intensively sampled in single-gene analyses. Initially, combinations of plastid and/or plastid and nuclear (invariably 18S) loci were analyzed (e.g., Graham and Olmstead 2000; Soltis et al. 2000), and mitochondrial gene sequences, such as *atp*1, *cox*1, and *mat*R, were also added (e.g., Parkinson et al. 1999; Qiu et al. 1999; Soltis et al. 1999; Duvall 2000).

Multigene studies avoid phylogenetic interpretations based on single gene histories, which might not reflect organismal phylogeny (Sanderson and Shaffer 2002; Duvall and Ervin 2004). Trees inferred from multiple genes in different genomes are based on large character sets and are less likely to be dominated by locus or genome-specific processes. Nonetheless, while multigene phylogenies have resolved a number of fundamental questions, they have not placed the monocots. For example, a three-gene study of 560 angiosperms that sampled one nuclear and two plastid loci placed monocots in a polytomy with eumagnoliids (Canellales, Laurales, Magnoliales, and Piperales sensu APG II; see Qiu et al. 2000) and Chloranthaceae (jackknife support, JS = 56%; Soltis et al. 1999). A four-gene study of one nuclear locus, one mitochondrial locus, and two plastid loci from 16 species retrieved the Piperales, sensu APG II, as the sister group to the monocots (bootstrap support, BS ≤ 50%; Duvall 2000). A more extensive analysis of one nuclear locus with two mitochondrial and two plastid loci from 97 angiosperms united monocots with Ceratophyllaceae, a taxonomically problematic family of aquatic paleoherb dicots (BS ≤ 50%; Qiu et al. 1999). A recent global analysis in which 26S data were added to the five genes analyzed by Qiu et al. (1999) also united Ceratophyllaceae with monocots, but this topology was not supported in bootstrap analysis (BS ≤ 50%; Zanis et al. 2003). A similar topology was inferred in an analysis of 17 slowly evolving plastid sequences from 16 exemplar angiosperms, but with only 42% BS (Graham and

Olmstead 2000). Another analysis of three slowly evolving mitochondrial genes plus the plastid *rbc*L and the nuclear 18S regions from 45 angiosperms united monocots with Laurales (BS ≤ 50%; Parkinson et al. 1999). Finally, a study of two plastid genes sampled from 349 species of angiosperms united monocots with the eumagnoliids sensu Qiu et al. (2000) again with BS ≤ 50% (Savolainen et al. 2000).

Causes of Ambiguity

Nearly all previous studies that have included monocots and their potential sister groups addressed broad and/or multiple goals, emphasizing the identification of basal angiosperm lineages as well as determining relationships within paleoherbs and within eudicots. These objectives guided sampling strategies, perhaps to the exclusion of data that would be relevant to the placement of monocots. Moreover, sampling in smaller studies was sufficiently different to prevent the direct comparison of results. And, due to technical limitations, the decision to add more genes conflicted with the potentially beneficial effects of adding taxa.

A more specific problem has been reliance on 18S as the nuclear marker. Monocots are consistently non-monophyletic in 18S gene trees. An early phylogenetic study of 263 base pairs (bp) of 18S from 21 tracheophytes placed eudicots within monocots (Troitsky et al. 1991). In studies with more taxa and characters, Acoraceae, the basal lineage of monocots in most other molecular trees, are separated from the monocot clade and Ceratophyllaceae, a potential monocot sister group, are nested within monocots. For example, in a study of 1164 aligned bp for 37 species, the 18S sequence of *Acorus calamus* was sister to sequences of Piperales; the remaining monocots were sister to *Saruma henryi* (Aristolochiaceae) (Bharathan and Zimmer 1995). An analysis of 1853 bp of 18S sequences from 59 angiosperms placed *A. calamus* in a polytomy with three other monocots, various paleoherb dicots, Magnoliales and eudicots (Nickrent and Soltis 1995). A more extensive analysis of 223 species placed *A. calamus* in a basal grade of dicots, again separated from the remaining 28 species of monocots in the analysis (Soltis et al. 1997). It was concluded that "... *Acorus* is anomalous among monocots ..." and that "... the 18S rDNA of *Acorus* should be resequenced and additional monocots should be added to the data set before the affinities of this enigmatic genus are addressed further ..." (Soltis et al. 1997; p. 21). Thus, increased sampling of 18S from relevant angiosperms has neither resolved a single monocot clade nor has it robustly placed them in angiosperm trees. The persistent non-monophyly of monocots in 18S trees is perplexing given the support for monocot monophyly in other data sets. There are several plausible explanations for anomalous placement, five of which are briefly considered below.

(1) The published 18S sequence of Acorus calamus *is erroneous.*—18S sequence identities of 99.1% between two different species (*A. calamus*—L24078 and *A. gramineus*—AF197584) produced in two different laboratories at different times substantiate the authenticity of these published sequences. Further confirmation comes from the high (99.9%) sequence identity between two sequences of *A. gramineus* obtained from different starting material in different laboratories and from the similarly high sequence identities of multiple cloned portions of 18S from *A. americanus* (Raf.) Raf. to the 18S sequence of *A. calamus* (Duvall and Ervin 2004). These results indicate that published 18S sequences from Acoraceae are authentic.

(2) Monocots are not monophyletic.—A second possibility is that 18S gene trees accurately reflect evolutionary relationships. However, the weight of morphological and molecular evidence argues against this hypothesis. An unambiguous morphological synapomorphy is the monocotyledonous embryo. The few dicot groups that have lost a cotyledon, have one vestigial cotyledon, or have two fused cotyledons are clearly separate derivations of the monocotyledous condition (Dahlgren et al. 1985; Tillich 1995). Other potential monocot synapomorphies, such as sieve-element plastids with cuneate protein crystals, atactosteles with numerous leaf traces diverging in parallel, and the "monocot" pattern of anther wall development (Davis 1966; Duvall 2001), are variously homoplasious. However, substantive additional evidence for the monophyly of monocots can be found in phylogenetic analyses of *rbc*L (e.g., Chase et al. 1993), phytochromes A and C (*PHY*A and *PHY*C; Mathews and Donoghue 1999, 2000), *rps*4 (Nadot et al. 1995), *ndh*F (Duvall 2000), and 17 plastid genes analyzed together (Graham and Olmstead 2000). Thus, the anomalous 18S phylogeny requires a different explanation.

(3) Insufficient taxon density.—Previously published 18S analyses with broad goals to determine relationships among major clades of angiosperms may have included too few taxa to resolve the monocots as monophyletic. To address this possibility, published and unpublished 18S sequences from 70 species, originally produced for seven different studies, were combined (Duvall and Ervin 2004). In this analysis of 18S sequences, monocots (including two species of Acoracae plus 13 other species emphasizing basal lineages) were analyzed together with species of other major groups sensu APG including: Amborellaceae and four other members of ANITA (basal grade on the flowering plant phylogeny composed of *Amborella* Baill., Nymphaeales and Illiciales–Trimeniaceae–*Austrobaileya* C. T. White; Qiu et al. 1999), Canellales (8 spp.), Ceratophyllaceae (1 spp.), Chloranthaceae (3 spp.), Laurales (14 spp.), Magnoliales (8 spp.), Nymphaeaceae (4 spp. plus *Cabomba* Aubl.), and Piperales (11 spp. including Aristolochiaceae, Saururaceae, Piperaceae, and *Lactoris* Phil.). In spite of the greater taxon density in this analysis, monocots were not monophyletic. Not only were Acoraceae positioned in a phylogenetically distant location from the other monocots, but Ceratophyllaceae were embedded within the monocots. The monophyly of monocots plus *Ceratophyllum* L., exclusive of *Acorus calamus* and *A. gramineus,* was well supported in Bayesian analyses with a posterior probability (PP) of 1.00 (Duvall and Ervin 2004). Thus, adding 18S sequences from potential sister lineages actually increased the support for the anomalous positions of Acoraceae and Ceratophyllaceae.

(4) Long branch attraction (LBA).—Acoraceae were found on a branch with a length in the upper 7% of all the branches in 18S gene trees (Duvall and Ervin 2004), suggesting that LBA might be an issue. Huelsenbeck (1997) proposed that

Table 1. Voucher information for taxa newly sequenced for this study.

Species	Loci sequenced	Voucher
Aquilegia canadensis L.	*rbc*L, *atp*1	*Duvall s. n.* (DEK)
Asarum arifolium Michx.	*ndh*F	*Kelly 672* (BH)
Asarum canadense L.	*PHYC*	*Mathews 487* (A)
Asparagus officinalis L.	*ndhF*	*Qiu 94063* (IND)
Buxus sempervirens L.	*PHYC*	*Mathews 472* (A)
Canella winterana (L.) Gaertn.	*ndh*F	*Qiu 90017* (NCU)
Carludovica palmata Ruiz & Pav.	*PHYC*, *ndh*F	*Qiu 97021* (IND)
Degeneria vitiensis I. W. Bailey & A. C. Smith	*ndh*F	*Miller 1189-63* (MO)
Euptelea polyandra Siebold & Zucc.	*PHYC*, *ndh*F	*Mathews 467* (DEK)
Hedycarya arborea J. R. Forst. & G. Forst.	*ndh*F	*Qiu 90028* (NCU)
Idiospermum australiense S. T. Blake	*ndh*F	*Qiu 91042* (NCU)
Joinvillea plicata Hook. f.	*atp*1	*Thien 84* (NO)
Joinvillea ascendens Gaudich. ex Brongn. & Gris	*PHYC*	*Moore 10438* (NY)
Knema latericia Elmer	*PHYC*, *ndh*F	*Qiu 91041* (NCU)
Lilium lancifolium Thunb.	*atp*1	*Duvall s. n.* (DEK)
Liriodendron tulipifera L.	*PHYC*, *atp*1	*Duvall* DEK000372
Meliosma squamulata Hance	*PHYC*, *ndh*F	*Qiu 99002* (Z)
Nymphaea odorata Aiton	*ndh*F	*Les s. n.* (CONN)
Platanus occidentalis L.	*PHYC*, *ndh*F	*Duvall s. n.* (DEK)
Pseudowintera axillaris Dandy	*ndh*F, *atp*1	*Mathews 412* (A)
Sabia swinhoei Hemsl.	*PHYC*	*Qiu 99003* (Z)
Sagittaria latifolia Willd.	*atp*1	*Duvall* DEK8-13-02
Sarcandra chloranthoides Gardner	*ndh*F	*Qiu 92002* (NCU)
Tofieldia calyculata (L.) Wahlenb.	*PHYC*, *ndh*F	*Qiu 97041* (IND)

LBA can be invoked when two conditions are met: first, long branches are clustered together in trees produced under methods sensitive to LBA but separated in trees produced under less sensitive methods; and second, when branches have been previously determined to be long enough to attract in simulated data sets. We found that only the first of these conditions was met in analyses of 18S. First, in analyses of a subset of 20 species, *Acorus calamus* was sister to the long branch taxon (LBT), *Triglochin maritimum* L. (Duvall and Ervin 2004), in maximum parsimony (MP) trees, but not sister to any LBT in parallel Bayesian inference (BI) trees. And, in simulation studies, when Acoraceae were forced into a sister group position with another LBT, such as *T. maritimum* or *Peperomia serpens* (Sw.) Loudon, they retained that association in about three-quarters of the simulations. However, Acoraceae did not cluster with other LBTs with which they had not been forced, nor did they associate with LBTs in more than 2% of analyses of simulated data sets when not so forced. Moreover, Acoraceae have not been found to be sister to other LBTs in previously published 18S gene trees nor in our larger 18S trees. If LBA was responsible for the anomalous phylogenetic position of Acoraceae in these trees, this family should frequently associate with other LBTs in simulated and actual maximum parsimony trees. Thus, it is difficult to attribute the paraphyly of monocots in 18S gene trees solely to LBA.

(5) Phylogenetic conflict reflects different underlying evolutionary histories.—18S trees that conflict with other trees could result from processes such as horizontal gene transfer, lineage sorting, or ancient hybridization among ancestral species. Although these processes are commonly perceived in population-level studies, the long-term consequences of events such as lineage sorting can be seen as macroevolutionary patterns even in long-diverged lineages (Satta et al. 2000; Duvall and Ervin 2004). Limited horizontal transfer of mitochondrial genes has been demonstrated in flowering plants (Bergthorsson et al. 2003), though horizontal transfer of nontransposable nuclear elements between eukaryotes may be rare (Graur and Li 2000). Differential lineage sorting from a polymorphic ancestral population may be more likely (Brower et al. 1996). Since nuclear ribosomal loci are highly duplicated in plants, their genomes may harbor paralogs with different histories, although concerted evolution tends to counteract this tendency (Zimmer et al. 1980). Although all the available evidence suggests that 18S diversity in *Acorus* is low to nonexistent (Duvall and Erwin 2004), historical 18S diversity remains a possibility. If multiple 18S paralogs existed in the ancestral population that gave rise to monocots and related dicot lineages, the clustering of *Acorus* with Piperales, and of other monocots with *Saruma* Oliv., might be indicative of differential sorting of 18S and/or biased gene conversion that led to retention of closely related paralogs in phylogenetically distant taxa.

Together these observations suggest that 18S sequences from Acoraceae are authentic and that the conflict between 18S and other gene trees with respect to relationships among monocots and dicots is real and should be further explored. Conflict among 18S and other trees does not appear to result merely from inadequate sampling. Thus, topological conflict among trees from different data sets is a plausible explanation of poorly supported nodes in multigene trees inferred from 18S and other data.

To explore this, we investigated the 5′ exon of *PHYC*. *PHYC* evolves more rapidly than plastid loci (Mathews et al. 1995) and thus may be useful for resolving short interior branches. Coding sequences of *PHYC* are highly conserved

Table 2. Taxa included in this study. GenBank accession numbers are given for each of four loci. *PHYC* sequences were determined for the species listed. Congeners are listed for other loci when different.

Species	*PHYC*	*ndh*F	*rbc*L	*atp*1
Acorus gramineus Soland.	AF190061	DQ356467	M91625	AF197622
			A. calamus L.	
Amborella trichopoda Baill.	AF190063	AF235046	L12628	AF197711
Aquilegia L. sp.	AF190067	AF130233	AY392755	AY394727
		A. bicolor Ehrh.	*A. canadensis* L.	*A. canadensis* L.
Aristolochia grandiflora Sw.	AF276713	DQ356468	L12630	AF197669
		A. gigantea Mart. & Zucc.	*A. macrophylla* Lam.	*A. macrophylla* Lam.
Asarum canadense L.	AY396705	AY394733	L14290	AF197671
		A. arifolium Michx.		
Asparagus falcatus L.	AF276715	AY394734	L05028	AF197713
		A. officinalis L.	*A. officinalis* L.	*A. officinalis* L.
Austrobaileya scandens C. T. White	AF190069	AF238052	L12632	AF197664
Buxus sempervirens L.	AY396706	AF241600	AF093717	AF197636
Calycanthus floridus L.	AF190073	AF123802	L14291	AF197678
Canella winterana (L.) Gaertn.	AF190075	AY394735	AJ131928	AF197676
Carludovica palmata Ruiz & Pav.	AY396707	DQ355787	AF19796	AF197707
Ceratophyllum demersum L.	AF276717	AF130232	D89473	AF197627
Chloranthus spicatus Mak.	AF190077	DQ356469	L12640	AF197668
		C. japonicus Siebold	*C. japonicus* Siebold	*C. multistachys* Pei
Degeneria vitiensis I. W. Bailey & A. C. Smith	AF190079	AY394736	L12643	AF293752
Dioscorea elephantipes (L'Hér.) Engl.	AF276721	AY007652	AF307461	AF197709
		D. bulbifera L.		*D.* L. sp.
Drimys winteri J. R. Forst. & G. Forst.	AF190081	AF123806	L01905	AF197673
Euptelea Siebold & Zucc. sp.	AY396708	AY394737	AY048174	AF197650
		E. polyandra Siebold & Zucc.	*E. pleiosperma* Hook. f. & Thoms.	*E. polyandra* Siebold & Zucc.
Hedycarya angustifolia A. Cunn.	AF190085	AY394738	L12648	AF197689
			H. arborea J. R. Forst. & G. Forst.	*H. arborea* J. R. Forst. & G. Forst.
Hedyosmum Sw. sp.	AF276723	DQ356470	L12649	AF197668
		H. arborescens Sw.	*H. arborescens* Sw.	*H. arborescens* Sw.
Houttuynia cordata Thunb.	AF190088	DQ356471	L08762	AF197632
Idiospermum australiense S. T. Blake	AF190090	AY394739	L12651	AF197680
Illicium oligandrum Merr. & Chun	AF276729	AF123808	L12652	AF197663
		I. parviflorum Michx. ex Vent.	*I. parviflorum* Michx. ex Vent.	*I. floridanum* Ellis
Joinvillea ascendens Gaudich. ex Brongn. & Gris	AY396709	U21973	L01471	AY394728
			J. plicata Hook. f.	*J. plicata* Hook. f.
Knema latericia Elmer	AY396710	AY394740	L12653	AF197697
Lactoris fernandeziana Phil.	AF190092	AF123809	L08763	AF197710
Lilium superbum L.	AF276733	AY007655	L12682	AY394729
				L. lancifolium Thunb.

Table 2. Continued.

Species	*PHYC*	*ndh*F	*rbc*L	*atp*1
Liriodendron tulipifera L.	AY396711	AF130230	AF190430	AY394730
Magnolia × *soulangeana* Hort. [ex Thieb.]	AF190095	AF107928 *M. tripetala* L.	AF206791 *M. tripetala* L.	AF197691 *M. tripetala* L.
Meliosma Blume sp.	AY396712	AY394741 *M. squamulata* Hance	AF206793 *M. veitchiorum* Hemsl.	AF197656 *M. squamulata* Hance
Nymphaea alba L.	AF190099	AY394742 *N. odorata* Aiton	M77034 *N. odorata* Aiton	AF197639 *N.* L. sp.
Piper nigrum L.	AF190101	DQ356472	L12660 *P. betle* L.	AF197630 *P. betle* L.
Platanus occidentalis L.	AY396713	AY394743	L01943	AF197655
Pseudowintera axillaris Dandy	AF276738	AY394744	AF093735 *P. colorata* (Raoul) Dandy	AY394731
Sabia swinhoei Hemsl.	AY396714	AJ236276	L12662 *Sabia* Colebr. sp.	AF197657 *Sabia* Colebr. sp.
Sagittaria L. sp.	AF190103	AY007657 *S. latifolia* Willd.	L08767 *S. latifolia* Willd.	AY394732 *S. latifolia* Willd.
Sarcandra glabra (Thunb.) Nakai	AF276742	AY394745 *S. chloranthoides* Gardner	L12663 *S. grandiflora* (Miq.) Subr. & Henry	AF197666 *S. chloranthoides* Gardner
Saruma henryi Oliv.	AF190105	DQ356473	L12664	AF197672
Smilax rotundifolia L.	AF276744	AF276018 *S. hispida* Muhl.	Z77310 *S. glauca* Walter	AF039251
Spathiphyllum wallisii Hort.	AF276746	AY007658 *S.* sp.	AJ235807	AF197706
Tofieldia calyculata (L.) Wahlenb.	AY396715	AY394746	AJ235798	AF197704
Trochodendron aralioides Siebold & Zucc.	AF190109	AF123812	L01958	AF197648

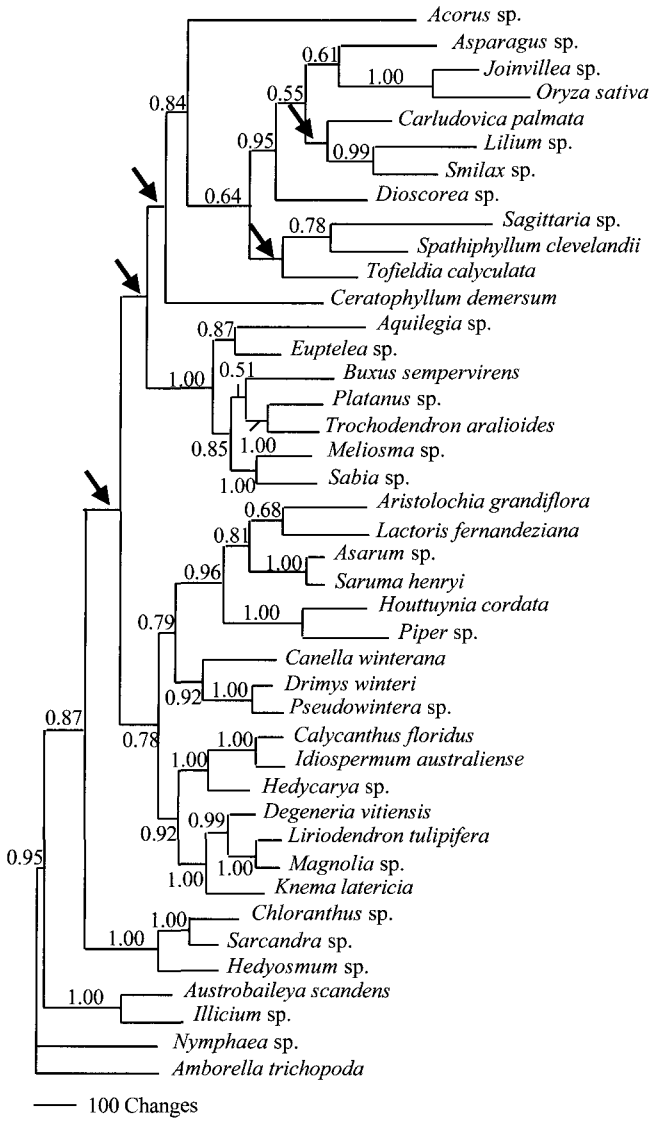

Fig. 1.—Single MP tree inferred from the combined analysis of data from four genes (*PHY*C, *rbc*L, *ndh*F, and *atp*1) and 42 taxa. Branch lengths are proportional to the number of steps along each branch. Bootstrap values appear along the branches. Arrows indicate nodes that are not supported on the bootstrap consensus tree.

across angiosperms and are easy to align (Mathews and Sharrock 1997; Mathews and Donoghue 1999, 2000); *PHY*C is also readily distinguishable from related phytochromes with locus-specific primers, and while taxon-specific duplications of *PHY*A and phytochrome B (*PHY*B) are known, none have been detected in *PHY*C (Mathews and Sharrock 1997; Mathews and Donoghue 2000).

METHODS

We analyzed a matrix of four loci (the 5′ exon of *PHY*C, mitochondrial *atp*1, plastid *rbc*L and the 5′ conserved region of plastid *ndh*F corresponding to coordinates 102216–103614 in the complete plastid genome of *Oryza sativa* L., GenBank accession NC001320) and 42 taxa. We sampled basal taxa from each major clade of angiosperms, and included the putative sister groups of the monocots, Magnoliales, Piperales, Ceratophyllaceae and Chloranthaceae. Voucher-specimen information for newly determined sequences is listed in Table 1. The complete list of sequences analyzed is listed in Table 2. *Amborella trichopoda* was designated as the outgroup. Three other taxa from the ANITA grade were included, as were representative Chloranthaceae (3), Ceratophyllaceae (1), eumagnoliids (16), eudicots (7), and monocots (11). Sequence determination followed standard methods (Mathews and Donoghue 1999; Duvall 2000). Localized hotspots prone to insertions/deletions (indels) were excluded from the phylogenetic analyses; sequence termini were truncated to reduce missing data. The 3′ variable region of *ndh*F was also excluded.

Maximum parsimony (MP), neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) methods were used, the first three as implemented in PAUP* vers. 4.0b10 (Swofford 2002) and the fourth as implemented in MrBayes vers. 3.0b4 (Huelsenbeck and Ronquist 2001). Heuristic MP analyses were performed using 100,000 random addition sequences and TBR swapping. MP bootstrap analysis (Felsenstein 1985) was conducted with 1000 bootstrap replicates, ten random addition sequences per bootstrap replicate, and TBR swapping.

The best-fit likelihood model for these sequence data was selected by using Modeltest vers. 3.06 (Posada and Crandall 1998), which selects the optimal model from 56 possibilities under the Akaike information criterion. The optimal model identified by Modeltest analysis was the general time-reversible (GTR) nucleotide substitution model with gamma-distributed among-site rate variation and allowing for heterogeneous rates across sites (Γ) and a proportion of invariable (I) sites (GTR + Γ + I). NJ analysis (Saitou and Nei 1987) was conducted under this model, again with 1000 bootstrap replicates. MP and NJ analyses failed to resolve, or showed only low bootstrap support, for the deeper branches in the tree. Further explorations of these data were conducted with BI and ML.

Both the BI and ML methods are based on the likelihood function. For comparative purposes, parallel BI and ML analyses were run on the same 29-taxon data subset of the four combined loci. A smaller subset was necessary to reduce the computational burden in the ML analysis. Parameters for the ML analysis were obtained from the Modeltest analysis. This analysis was performed under the heuristic search option with a single random addition search. The BI analysis was conducted as specified below.

BI analyses were conducted on both the single-gene and the combined data of the 42-taxon matrix. Combined analyses were also conducted on various taxon subsets (see below). BI uses a specified model of evolution to estimate PP, the probability of a tree given the sequence data for a set of taxa. The PP was calculated using a Metropolis-coupled Markov Chain Monte Carlo method that samples trees based on their likelihood values relative to other trees. BI analyses were performed under the GTR model (N_{ST} = 6) with a proportion of invariable sites and among-site rate variation for the remaining sites drawn from a Γ distribution (rates = invgamma). No prior probability distribution was assumed so that all trees were given equal weight a priori. In combined analyses, sequences were partitioned by gene- and site-specific rates were allowed to vary across partitions

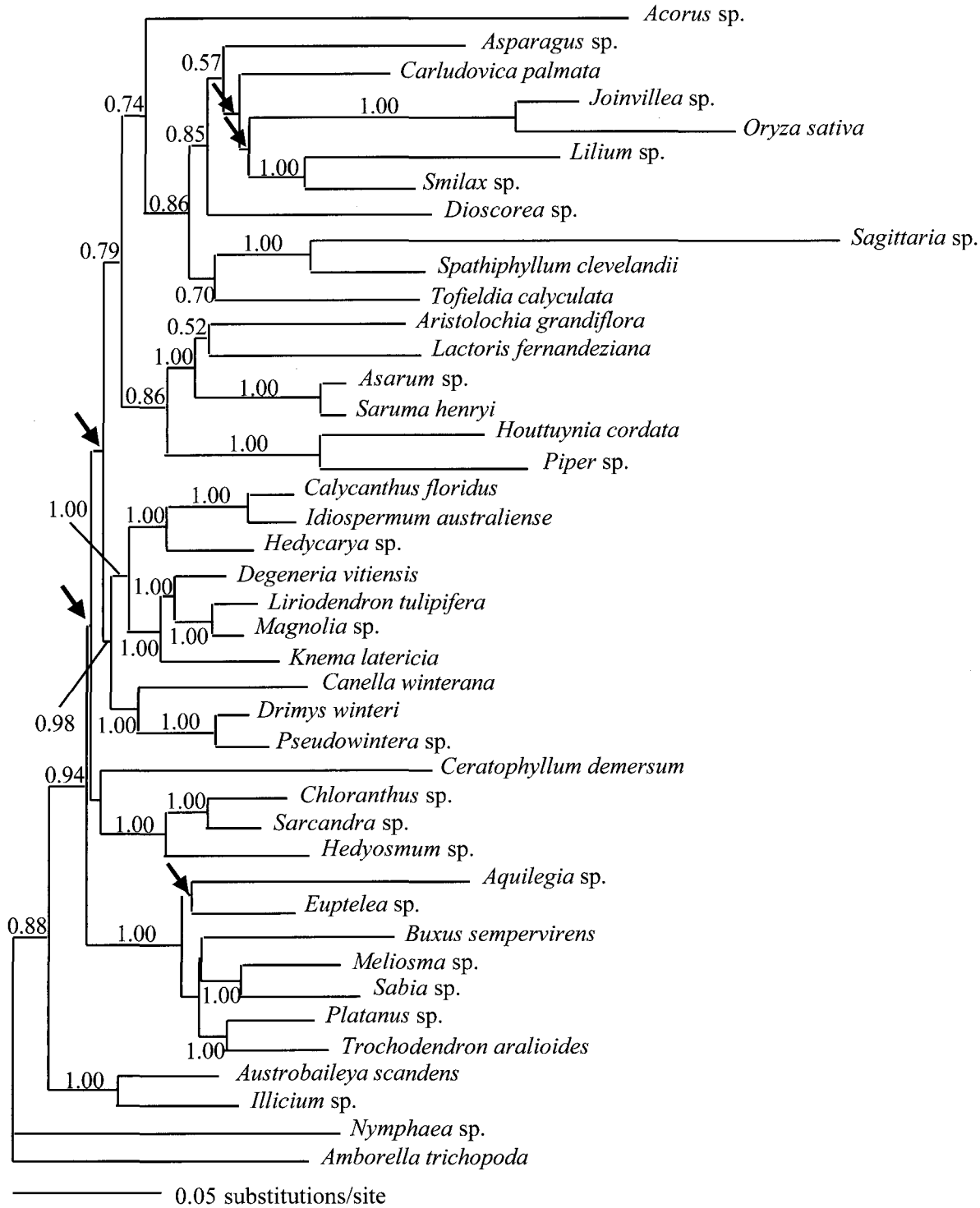

Fig. 2.—NJ tree inferred from the analysis of the same data matrix analyzed in Fig. 1. Branch lengths are proportional to the number of steps along each branch. Bootstrap values appear along the branches. Arrows indicate nodes that are not supported on the bootstrap consensus tree.

(ratepr = variable). Default settings were used for other prior probability parameters. All BI analyses were executed for 1,000,000 generations with trees sampled every 80 generations. The first 2501 trees were discarded, after which improvement in the range of log-likelihood values was not observed.

Analyses were performed under these conditions with 20 different taxon subsets of the complete data matrix to explore how sampling affected topology, and with the further effect that each analysis would have a different starting tree.

RESULTS AND DISCUSSION

GenBank accession numbers for the sequences determined for this study are listed (Table 2). Twelve indels, ranging in

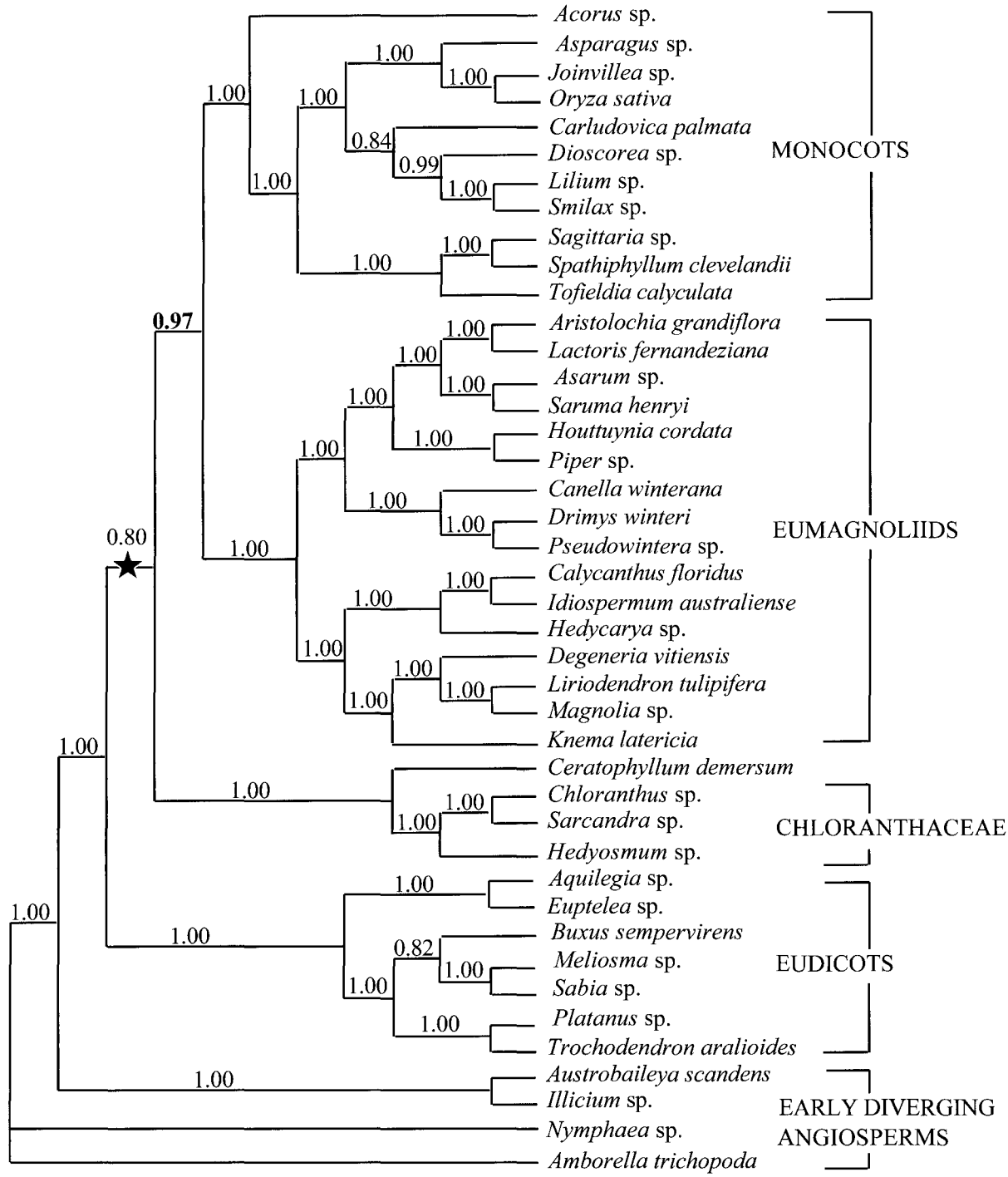

Fig. 3.—Bayesian consensus tree of the last 10,000 trees (of 12,501) inferred from the same data matrix analyzed in Fig. 1–2. PP values are indicated along the branches. The position of one informative indel in *PHYC* is indicated with a star. Note that the node uniting monocots and eumagnoliids (PP = 0.97) is supported in the six trees of the 0.90 credible set.

size from 3 to 18 bp, were observed, distributed among *ndh*F, *PHYC*, and *atp*1. Nine of these were autapomorphic. Two more each arose in parallel lineages that were unrelated in all our analyses. One three-bp insertion in *PHYC*, located after position 1629 in the complete reference sequence AF141942 from *Oryza sativa*, was found in all taxa excluding the members of eudicots and the ANITA grade sampled here. After exclusion of these indels and the sequence termini, the remaining conserved regions were unambiguously aligned to 5024 sites. The aligned lengths of the sequences analyzed were 1158 bp (*PHYC*), 1230 bp (*atp*1), 1349 bp (*rbc*L), and 1287 bp (*ndh*F).

MP analysis of the 42-taxon × 5024-character matrix produced a single, most-parsimonious tree of 10,342 steps (see Fig. 1 for MP bootstrap results). The results of the NJ bootstrap analysis of the same data matrix are presented in Fig.

Table 3. 0.95 credible set of ten trees for the 42 taxon BI analysis, in decreasing order of overall posterior probability (PP). The first six trees comprise the 0.90 credible set. Monocot placement for each tree is indicated and remains stable through the eighth tree. Note the rapid decline in PP across the 0.95 credible set.

Tree	Overall PP of tree	Cumulative PP	Monocot placement
1	0.538	0.538	((Monocots, Eumagnoliids), Chloranthaceae), . . .
2	0.119	0.657	((Monocots, Eumagnoliids), Eudicots), . . .
3	0.110	0.767	((Monocots, Eumagnoliids), Eudicots), . . .
4	0.093	0.861	((Monocots, Eumagnoliids), Chloranthaceae), . . .
5	0.033	0.893	((Monocots, Eumagnoliids), Eudicots), . . .
6	0.026	0.919	((Monocots, Eumagnoliids), Chloranthaceae), . . .
7	0.018	0.937	((Monocots, Eumagnoliids), Chloranthaceae), . . .
8	0.006	0.943	((Monocots, Eumagnoliids), Chloranthaceae), . . .
9	0.006	0.949	((Monocots, Chloranthaceae), Eumagnoliids), . . .
10	0.006	0.955	(Monocots, (Chloranthaceae, Eumagnoliids), . . .

2. The ML analysis of the 29-taxon subset produced a tree with a −lnL score of 42,365.85267 (results not shown) in which monocots were in an identical position as in the parallel BI analysis of the same taxon subset (see below). The BI analysis of the 29-taxon subset matrix was completed in somewhat less than 40% of the 122 hr of computer time required for the ML analysis. The consensus tree of the 42-taxon BI analysis of the entire data matrix is presented in Fig. 3.

Three major clades were resolved in all analyses (BI, ML, MP, and NJ) of the combined data: Chloranthaceae, eudicots, monocots, and a clade of eumagnoliids were resolved in all but the NJ tree. In the following discussion, parenthetically indicated support values are BS for MP and NJ analyses, and PP for BI analyses. Chloranthaceae were strongly supported as monophyletic in all analyses (MP: 100%; NJ: 100%; BI: 1.00). The position of this clade varied across the BI, MP, and NJ trees (see Fig. 1–3) and recent studies are not in agreement on the placement of the family (e.g., APG II 2003; Hilu et al. 2003). However, in both BI and NJ analyses the immediate sister group to Chloranthaceae was *Ceratophyllum demersum,* and this result was strongly supported in the BI tree (1.00), although weakly supported in the NJ tree (≤50%).

Ceratophyllum L. is the sole genus in the family Ceratophyllaceae, comprising about six aquatic species of cosmopolitan distribution. Before the advent of molecular phylogenetic tools, this family was rarely placed far from the water lilies (Nymphaeales). Cronquist (1981) treated them as the most specialized and reduced member of this group, stating that there was "no doubt about" the link between *Ceratophyllum* and the water lily genus *Cabomba.* He inferred this link from the morphology of the submerged leaves, which he considered similar in the two genera. However, after a careful study of morphology, anatomy, and embryology, Les (1988) concluded that Ceratophyllaceae shared no close relationship to any Nymphaeales. He placed them in their own order and suggested that they had arisen prior to the divergence of monocots from dicots.

Previous molecular phylogenetic studies are equivocal regarding the placement of Ceratophyllaceae. Three studies of plastid genes suggest either that Ceratophyllaceae diverged very early in angiosperm evolution (Les et al. 1991; BS ≤ 50%) or that the family is in a sister position to all other angiosperms (Chase et al. 1993; Savolainen et al. 2000; BS ≤ 50%). Multigene analyses have either weakly united the family with monocots (BS ≤ 50% in both Graham and Olmstead 2000; Qiu et al. 2000; Zanis et al. 2003) or with eudicots (jackknife support, 53% in Soltis et al. 2000). In light of results from multigene analyses, it is interesting to note that *mat*K alone unites Ceratophyllaceae with eudicots in a moderately to well-supported clade (Hilu et al. 2003). This is consistent with the suggestion that conflict among 18S and other gene trees decreases support for monocot placement in multigene analyses. We are aware of only two studies that suggested an association with Chloranthaceae. The plastid rDNA ITS trees of Antonov et al. (2000) unite *Ceratophyllum* and *Chloranthus* Sw. with NJ support bootstrap values ranging from 83 to 88%. The mitochondrial *atp*1 tree of Bergthorsson et al. (2003) unites *Ceratophyllum* and *Sarcandra* Gardner (MP: 61%; BI: 1.00).

Ceratophyllaceae and Chloranthaceae have a suite of characters shared by early-diverging angiosperms that are consistent with their divergence soon after the ANITA grade, including stamens that are not differentiated into anther and filament, anthers with apically extended connectives and two disporangiate bulging thecae, and strongly ascidiate carpels that are sealed by secretion (Endress 1984, 2001). Similarities that suggest the existence of synapomorphies include leaves that are opposite (at first plumule node) or whorled in Ceratophyllaceae and opposite or decussate in Chloranthaceae; leaves with teeth; pollen grains that are inaperturate in Ceratophyllaceae and either inaperturate or aperturate in Chloranthaceae; monocarpellate gynoecia, highly reduced flowers that are strictly unisexual in Ceratophyllaceae and either uni- or bisexual in Chloranthaceae; and single, pendent orthotropous ovules. Although most of these character states might be symplesiomorphic or convergent, they bear re-examination in light of the potentially close relationship of these two families.

Eudicots were represented by taxa previously identified as basal members of the clade (Buxaceae, Sabiaceae, Trochodendraceae, Proteales, and Ranunculales). These basal eudicots were also resolved with uniformly strong support (BI: 1.00; MP: 100%; NJ: 100%), but the position of this clade was weakly supported (Fig. 1–3).

A clade designated as the "eumagnoliids" has been resolved as monophyletic in several molecular phylogenetic studies (Mathews and Donoghue 1999, 2000; Qiu et al. 1999, 2000; Barkman et al. 2000; Graham and Olmstead

2000). The group includes Canellales, Laurales, Magnoliales, and Piperales sensu APG II. Unequivocal morphological synapomorphies have not yet been identified for this group in which numerous plesiomorphic characters are found (Doyle and Endress 2000). The eumagnoliids were also resolved here in all but the NJ analysis with moderate to strong support (MP: 78%; BI: 1.00). In the NJ tree, Piperales (86%) were excluded from eumagnoliids (68%) and were united with monocots (79%; see Fig. 2).

Monocots were monophyletic in all trees from combined analyses, with support values of 74% (NJ), 84% (MP), and 1.00 (BI). *Acorus* was sister to the remaining monocots in all of these analyses with varying support (MP: 64%; NJ: 86%; BI: 1.00). In the MP tree, monocots were weakly united with *Ceratophyllum demersum* (BS $\leq$ 50%). The next lineage, basal to this group, was the eudicot clade (Fig. 1). In the NJ analysis, monocots were weakly paraphyletic with eumagnoliids (BS $\leq$ 50%) and moderately supported in a sister group position with Piperales (BS = 79%) with the next diverging clade comprising the remaining eumagnoliids (Canellales, Laurales, and Magnoliales). In contrast to these weakly supported positions, the ML and all 20 BI analyses consistently united monocots with the entire cluster of eumagnoliids. Support values in the BI trees for this monocot placement generally rose with increasing sample density, and stabilized at a minimum value of 0.97 (Fig. 3; PP values of 0.98–1.00 for the monocots/eumagnoliids clade have been obtained in other BI analyses, depending on sampling (not shown). In only one previous study were monocots placed as the sister clade to eumagnoliids with similarly strong support; a recent single-gene BI analysis of *atp*1 (Bergthorsson et al. 2003; PP = 1.00).

BI, as applied to phylogenetic analysis, is a relatively new method (Mau et al. 1999); Karol et al. (2001) reported one of its first uses in plant phylogenetics. Several recent papers compare PP values with measures of nonparametric bootstrap support (Suzuki et al. 2002; Wilcox et al. 2002; Alfaro et al. 2003; Douady et al. 2003). Some claim that that PP values are "excessively liberal" (Suzuki et al. 2002), while others suggest that PP values are ". . . much better estimates of phylogenetic accuracy" (Wilcox et al. 2002). Note that Wilcox et al. (2002), who evaluated PP values with simulation studies, concluded that a PP threshold of 0.95 was an indication of "significant support." Our conservative interpretation is that a PP $\geq$ 0.95 is an indication of at least "moderate" support.

Overall results from a BI analysis may be summarized as "credible sets" of trees, in which the trees are added in order of decreasing probability until some cumulative probability threshold is reached. Huelsenbeck et al. (2002) suggested that a relatively small credible set of largely similar trees would thus indicate strong support. The 0.90 credible set for the 42-taxon BI analysis (Fig. 3) contains only 6 trees (out of 10,000), and the differences between these trees are found within the well-defined clades. Monocot placement is stable until the last two trees of the ten-tree 0.95 credible set; these two trees have an associated posterior probability of only 0.006 (Table 3). The four single-gene BI analyses (not shown) had much larger credible sets with an average of 5380 trees in the 0.95 credible set, perhaps because each single-gene data set has less phylogenetic information than do the same sequences combined together. This observation is consistent with the failure of the single-gene BI analyses to resolve all of the major clades found by the combined analyses with at least moderate support. For example, in the single gene BI analysis of *atp*1, the monophylies of Chloranthaceae and of eudicots were weakly supported (PP = 0.52 and $\leq$ 0.50, respectively).

Multiple BI analyses of different taxon subsets for all four loci indicated the specific effects of sampling on phylogenetic topology (results not shown). Analyses that included taxonomically isolated species resulted in larger credible sets. For example, the addition of *Joinvillea* Gaudich. ex Brongn. & Gris sp. in the absence of other commelinoid monocots increased the size of the 0.95 credible set from 63 to 206 trees due to weakly supported alternative placements for this isolated species. In other cases, increasing representation within well-defined clades had the affect of stabilizing the topology. The addition of representative Laurales to other eumagnoliids (Canellales, Magnoliales, and Piperales) resulted in a moderately supported association between monocots and eumagnoliids with as few as 22 taxa. Monocot placement remained stable after the addition of Laurales, and was not altered by the inclusion or exclusion of various eudicots, Chloranthaceae or *Ceratophyllum,* although excluding the basal monocots (*Acorus* and *Sagittaria*) reduced the PP of the eumagnoliid/monocot clade from 0.97 to 0.89.

In conclusion, we found that analyses of a four-gene matrix excluding 18S rDNA and including *PHY*C yielded a well-supported clade uniting monocots with eumagnoliids. The measurably better support for monocot placement in our phylogeny suggests that at least some of the ambiguity surrounding the position of monocots in previous multigene analyses may have resulted from the conflict between 18S and other gene trees. Our results also imply a sister group relationship between Ceratophyllaceae and Chloranthaceae. The comparative clarity of this result might likewise be attributed to the exclusion of 18S and the inclusion of *PHY*C. Nevertheless, we believe that it would be premature to make conclusions about the stability of our results for these small divergent clades, in part because of the relationship between stability and sampling that we noted above. A recent, taxon-dense analysis of *mat*K placed Ceratophyllaceae and Chloranthaceae in different but well-supported positions, as sisters to eudicots and monocots, respectively, thus also suggesting a different monocot sister group (Hilu et al. 2003). Our study and the analysis of *mat*K represent contrasting strategies. We sampled four genes from a smaller number of taxa emphasizing basal members of major clades; Hilu et al. (2003) sampled one gene from many taxa, including both basal and many derived taxa. Perhaps neither of these strategies is adequate for understanding the evolution of monocots. Rather, it is likely that nuclear, plastid, and mitochondrial phylogenies, each inferred from many genes sampled from the same large number of taxa, will be needed to fully understand both genome and organismal evolution at this key divergence in the history of angiosperms.

Note added in proof.—Our phylogenetic analyses were performed prior to the Monocots III Conference (Mar 2003) and were completed for this paper later that year. Bergthorsson et al. (Dec 2004, *Proc. Natl. Acad. Sci. U.S.A.* **101**: 17747–17752) subsequently found that the *atp*1 sequence we

used for the outgroup, *Amborella trichopoda* (GenBank # AF197711), was likely from a horizontal gene transfer event from an unknown eudicot donor. A second copy of *atp*1 from this species (# AY009407), which was banked later, differed from the first by 57 substitutions across 1239 aligned base pairs of overlap. A BLAST search of this sequence targeted other early dicot *atp*1 subjects as expected, whereas a BLAST search of the AF197711 sequence targeted eudicots.

To see the effect of this sequence on our overall results, an amended BI analysis of the 42 taxon by 5024 base pair matrix was performed substituting the AY009407 sequence for the anomalous sequence of *Amborella* with other parameters as before. The topology of the majority rule consensus BI tree was unchanged. The monocots were still united with eumagnoliids (PP = 0.96 instead of 0.97; Fig. 3). Three other PP values for weakly supported nodes differed, two by ± 0.01 each. The greatest change was that supporting the position of the Ceratophyllaceae/Chloranthales clade (PP = 0.63 instead of 0.80). No other PP values were altered. The proportionate contribution of the outgroup *atp*1 sequence to the analysis did not otherwise affect the outcome of the BI analysis on which our conclusions were based.

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