INTRODUCTION

The past 20 years have witnessed significant progress in our understanding of the phylogeny of basal angiosperms from analyses of molecular and nonmolecular data (Dahlgren and Bremer 1983; Donoghue and Doyle 1989; Locote and Stevenson 1991; Martin and Dowd 1991; Hamby and Zimmer 1992; Taylor and Hickey 1992; Chase et al. 1993; Qiu et al. 1993, 1999, 2000, 2001; Soltis et al. 1997, 2000; Nandi et al. 1998; Hoot et al. 1999; Mathews and Donoghue 1999, 2000; Parkinson et al. 1999; Renner 1999; Soltis et al. 1999a; Barkman et al. 2000; Doyle and Endress 2000; Graham and Olmstead 2000b; Savolainen et al. 2000; Niekent et al. 2002; Zanis et al. 2002, 2003; Borsch et al. 2003; Hilu et al. 2003; Löhne and Borsch 2005). Specifically, it has become increasingly clear that Amborella, Nymphaeaceae, and Austrobaileyales (sensu APG II 2003) represent the earliest-diverging lineages of extant angiosperms. Furthermore, the magnoliids (sensu APG II 2003; see Qiu et al. 1993 for a review of the history of this term) have been identified as a monophyletic group in some analyses (Qiu et al. 1999, 2000; Zanis et al. 2002, 2003; Hilu et al. 2003), but their monophyly (Savolainen et al. 2000; Soltis et al. 2000) and especially relationships among their member orders (Magnoliales, Laurales, Piperales, and Canaellales) need further evaluation...
and resolution. Finally, all angiosperms excluding Amborella, Nymphaeaceae, and Austrobaileyales can be divided into five clades: Ceratophyllum, Chloranthaceae, magnoliids, monocots, and eudicots (tricolpates sensu Judd and Olmstead 2004; see also Walker and Doyle 1975; Crane 1989; Donoghue and Doyle 1989; Doyle and Hotton 1991; Chase et al. 1993). Relationships among these five lineages, however, are best interpreted as unresolved at present because analyses with different taxon and character-sampling schemes and phylogenetic methods have produced conflicting topologies that are generally only weakly supported (Barkman et al. 2000; Soltis et al. 2000; Zanis et al. 2002, 2003; Hilu et al. 2003).

Despite progress, more work is needed to further clarify relationships among basal angiosperms. In this study, we add sequence data of four new genes to a five-gene matrix assembled earlier (Qiu et al. 1999, 2000) and conduct parsimony, Bayesian, and maximum likelihood (ML) analyses to address several issues. First, we attempt to show that placement of Amborella, Nymphaeaceae, and Austrobaileyales at the base of angiosperm phylogeny is free of any analytical artifact. This is especially important in light of recent analyses of the entire plastid genome sequences of Amborella and Nymphaea that do not support them as basalmost angiosperms (Goremykin et al. 2003a, 2003b, 2004; but see Soltis and Soltis 1998; Soltis et al. 1999; Stefanovic et al. 2004). Second, we aim to evaluate the monophyly of magnoliids and to resolve the relationships among their members: Magnoliolae, Laurales, Piperales, Canellales. Finally, we wish to resolve relationships among Ceratophyllum, Chloranthaceae, magnoliids, monocots, and eudicots.

**Material and Methods**

We included 100 terminals from 98 genera, representing all major lineages of gymnosperms and basal angiosperms. Acorus and Ceratophyllum were the only two genera for which two species each were sampled. Only two families of basal angiosperms were not included, Gonospermaeae (Renner 1999) and Hydnoraceae (Nickrent et al. 2002), because of many missing data entries. Most of the terminals consist of sequences derived from a single species (and frequently the same DNA sample) and occasionally from different species of the same genus (tables 1, 2). Eight gymnosperms covering all four extant lineages were used as outgroups.

The four new genes added in this study are: plastid matK (a group II intron-encoded maturase), mitochondrial SSU (small subunit) and LSU (large subunit) rDNAs, and nuclear 26S rDNA. With the five genes from our earlier analyses (mitochondrial atp1 and matK, plastid atpB and rbcL, and nuclear 18S rDNA), the total of nine genes used in this study represents a sampling of a large number of characters from each of the three plant genomes. Furthermore, these genes encompass diverse functions, including energy metabolism, carbohydrate synthesis, RNA processing, and protein synthesis.

DNA extraction and sequencing methods follow Qiu et al. (2000). All primer sequences used for amplifying and sequencing the genes are available from the corresponding author on request. All sequences of mtLSU were newly generated in this study, whereas approximately half of the sequences were generated by us for mtSSU, matK, and nuclear 26S rDNA. For the five genes used in Qiu et al. (1999), several new sequences were produced to fill the missing entries in that matrix. The orthologous atp1 was used to replace the copy we obtained earlier from Amborella (Qiu et al. 1999, 2000), which has been shown to be a xenolog horizontally transferred from an asterid (Barkman et al. 2000; Berghthorsen et al. 2003). For all nine genes we have taken sequences from GenBank when appropriate. Detailed source information for all sequences and correction to errors in table A1 of Qiu et al. (2000) are provided in tables 1 and 2. Of all taxa and all genes, only four taxa have missing data in one or two genes: Metasequoia (mtSSU and matR), Hortonia (matR), and Dioscorea and Myristica (nu26S) (tables 1, 2). Eight of the nine genes (all except mtSSU) were aligned using Clustal X (Thompson et al. 1997). Because of extraordinary length variation in several regions of mtSSU, this gene was manually aligned with the alignment editor AE2 (developed by T. Macke; Larsen et al. 1993). Although these regions typically had minimal sequence identity that could not be aligned based on sequence alone, they usually had similar structural elements that facilitated the alignment of these sequences. In addition, all of the computer-generated alignments were manually adjusted with the MacClade 4.05 (Maddison and Maddison 2002) alignment editor. All of the aligned positions were used in the phylogenetic analyses. We also eliminated the positions in regions with significant length variations in the four rDNAs from the phylogenetic analyses of the nine-gene matrix. These latter analyses yielded results not substantially different from those presented here (data not shown).

Three series of analyses were performed to address various issues. First, two separate matrices were assembled to reconstruct the overall phylogeny of basal angiosperms, one consisting of all nine genes and the other of five protein-coding genes. The decision to make a separate matrix using the five protein-coding genes was based on the following considerations: (1) all positions within the protein genes should evolve more independently than those of rDNAs, many of which evolve in a coupled fashion due to base pairing in stem regions in these genes (Soltis and Soltis 1998; Soltis et al. 1999b; O. Dombrovska and Y.-L. Qiu, unpublished data); (2) the protein-coding genes generally show fewer problems of paralogy and xenology compared to nuclear 18S and 26S rDNAs, for which nonorthologous copies were occasionally encountered; and (3) the protein-coding genes are free of alignment uncertainties compared to two mitochondrial rDNAs, which exhibit extraordinary length variations caused by insertions and deletions in a few regions. The parsimony, Bayesian, and maximum likelihood (ML) analyses were implemented separately on both matrices. To evaluate the informativeness of the two nuclear rDNAs further, the five-protein-gene matrix was combined with 18S and 26S rDNAs sequentially to form two more matrices. Only parsimony bootstrap analyses were conducted on these two matrices.

Second, three separate genome-specific matrices were constructed to address whether placement of Amborella, Nymphaeaceae, and Austrobaileyales as sisters to all other angiosperms is supported by data from the plastid, mitochondrial, and nuclear genomes separately. This type of analysis has only been conducted occasionally (Mathews and
Donoghue 1999, 2000; Graham and Olmstead 2000b; Savolainen et al. 2000; Zanis et al. 2002). A robust understanding of organismal phylogeny should be based on evidence from each of the three plant genomes (Qiu and Palmer 1999) except in cases of hybridization and horizontal gene transfer. Only parsimony bootstrap analyses were conducted on these data sets.

Third, we investigated the types of substitutions that provided phylogenetic signal for identifying *Amborella*, Nymphaeaceae, and Austrobaileyales as the earliest-diverging lineages of extant angiosperms. For an issue as critical as the rooting of angiosperm phylogeny, merely having high bootstrap numbers from an analysis is not enough to gain confidence in the result (Soltis et al. 2004). Some poorly understood molecular evolutionary phenomena, such as RNA editing (Steinhauser et al. 1999; Kugita et al. 2003; Dombrovskia and Qiu 2004) and GC-content bias (Steel et al. 1993), both of which can occur in a genome-wide, lineage-specific fashion, can generate substitutions that lead to spurious groupings in phylogenetic analyses. Hence, it is important that we understand the types of substitutions that are behind those high bootstrap percentages. We examined the nine-gene matrix visually and identified the sites that contain apparently synapomorphic changes that separate gymnosperms-Amborella-Nymphaeaceae-Austrobaileyales from all other angiosperms. Sites were classified as apparently synapomorphic if they contained the same nucleotide in at least two of the four gymnosperm lineages (cycads, Ginkgo, conifer II (non-Pinaceae conifers), and Gnetales + Pinaceae; Bowe et al. 2000; Chaw et al. 2000) and at least two of the three basal angiosperm lineages (*Amborella*, Nymphaeaceae, and Austrobaileyales) but had a different and generally invariable nucleotide in all other angiosperms (hence a synapomorphy for euangiosperms, sensu Qiu et al. 1999). We then performed both a most parsimonious tree search and a parsimony bootstrap analysis with these sites removed to verify our identification. These synapomorphic substitutions were finally checked to determine if they could have been generated by RNA editing or GC-content bias. In addition, codon position and type of change (transition vs. transversion) were noted for these substitutions.

These last two series of analyses were designed to complement the analyses we performed earlier (Qiu et al. 1999, 2000, 2001), to ensure that the placement of *Amborella*, Nymphaeaceae, and Austrobaileyales as basal lineages is indeed based on historical signal recorded in the multiple genes from all three plant genomes rather than the result of yet poorly understood analytical artifacts. These analyses are particularly relevant in the ongoing debate over whether *Amborella* and Nymphaeae are basal angiosperms (Goremykin et al. 2003a, 2003b, 2004; Soltis et al. 2004; Soltis and Soltis 2004; Stefanovic et al. 2004).

In parsimony searches we used equal weighting for all positions and character-state changes using PAUP* 4.0b10 (Swofford 1998). When searching for the shortest trees, a heuristic search was conducted using 1000 random taxon-addition replicates, one tree held at each step during stepwise addition, TBR branch swapping, steepest descent option off, MulTrees option on, and no upper limit of MaxTrees. For bootstrap analyses, 1000 resampling replicates were performed (except for the matrix of five protein genes plus two nuclear rDNAs where 5000 resampling replicates were used) with the same tree search procedure as described above except with simple taxon addition and the steepest descent option on.

For Bayesian and ML analyses, the optimal models of sequence evolution for the nine-gene and five-protein-gene data sets were estimated using ModelTest 3.6 (Posada and Crandall 1998) and DT-ModSel (Minin et al. 2003). The general time-reversible model (Rodriguez et al. 1990) including parameters for invariant sites and rate variation (GTR + I + Γ) best fits both data sets and was used to conduct the analyses.

Bayesian analyses were performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001). For the nine-gene matrix, the data were partitioned according to codon positions (first, second, and third, for protein genes only), genomes (plastid, mitochondrial, and nuclear), and gene types within a genome (rRNA vs. protein genes). For the five-protein-gene matrix, the data were partitioned according to codon positions and genomes. Calculations of likelihood for searches of both matrices were implemented under the GTR + I + Γ model of sequence evolution, assuming different stationary nucleotide frequencies. The posterior probability (PP) was estimated by sampling trees from the PP distribution using Metropolis coupled Markov Chain Monte Carlo methods. Two and four chains of 5,000,000 generations were run for the nine-gene matrix and five-protein-gene matrix, respectively. Chains were sampled every 100 generations. Likelihood scores converged on a stable value after 500,000 generations (the burn-in of the chain), and calculations of PP were based on the trees sampled after this generation.

Maximum likelihood analyses were performed separately on the nine-gene and five-protein-gene data sets using PHYML version 2.4.4 (Guindon and Gascuel 2003) under the optimal model of sequence evolution. For both data sets, the GTR + I + Γ model was implemented with parameter values for the proportion of invariant sites (nine-gene = 0.19, five-gene-protein = 0.21) and the gamma distribution (nine-gene = 0.43, five-gene-protein = 0.68) as estimated by ModelTest 3.6 and DT-ModSel. The optimal rate of nucleotide substitution and transition/transversion ratios was estimated from the data during ML searches. Maximum likelihood support values were similarly estimated from 100 bootstrap replicates in PHYML.

**Results**

For the nine-gene data set, which contained 26,990 aligned nucleotides, two islands with two and four shortest trees (length = 51,834 steps; consistency index [CI] = 0.47; retention index [RI] = 0.57) were found 259 and 315 times, respectively, out of 1000 random taxon-addition replicates in the parsimony search. One of the six trees is shown (fig. 1), with the nodes that are not present in the strict consensus of all six trees indicated by asterisks.

For the five-protein-gene data set, which contained 9351 aligned nucleotides, a single island of two shortest trees (length = 18,839 steps; CI = 0.42; RI = 0.59) was found in all 1000 random taxon-addition replicates in the parsimony search. One of the two trees is shown (fig. 2), with the nodes
**Table 1**

Vouchers, Contributors, GenBank Accession Numbers, and References for the Sequences Used in This Study

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<th>Family and species</th>
<th>mt-SSU rDNA</th>
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<td>K.W. Hilm, K. Müller, and T. Borsch, unpublished manuscript; Borsch 3460, BONN AF543722 (KH)</td>
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Qiu 92007; Kew; AJ966790
Qiu 92007; LL/YQ; DQ008628
Whalen 132, NSW; JL/LL/YQ; DQ008680
Whalen 132, NSW; OD/FBQ/YQ; DQ008776
Whalen 132, NSW; Kew; AJ966791
Whalen 98109; OD/YQ; DQ008630

Daphnandra micrantha (Tul.) Benth.
Qiu 92007; JL/LL/YQ; DQ008681
Qiu 92007; OD/FBQ/YQ; DQ008827
Hilu et al. 2003; Borsch 3464
BONN AF543726 (TB)
Zanis et al. 2003; AY095452

Doryphora sassafras Endl.
Qiu 98109; JL/LL/YQ; DQ008682
Qiu 98109; OD/FBQ/YQ; DQ008777
Hilu et al. 2003; Borsch 3409
BONN AF542568 (TB)

Austrobaileyaceae:
Austrobaileya scandens C. T. White
Parkinson et al. 1999; AF193988
Qiu 90303; OD/FBQ/YQ; DQ008828
Hilu et al. 2003; Borsch 3464
BONN AF543726 (TB)

Berberidaceae:
Mahonia bealei (Fortune) Carr.
Qiu 74; JL/LL/YQ; DQ008683
Qiu 74; OD/FBQ/YQ; DQ008754
Hilu et al. 2003; Borsch 3405
BONN AF542585 (TB)
Qiu 97057; OD/YQ; DQ008613

Mahonia japonica
Qiu 92003; JL/LL/YQ; DQ008684
Qiu 92003; OD/FBQ/YQ; DQ008755
Hilu et al. 2003; Borsch 3393
BONN AF542586 (TB)
Qiu 92003; OD/YQ; DQ008614

Podophyllum peltatum L.
Qiu 90303; OD/FBQ/YQ; DQ008743
Qiu 97057; OD/FBQ/YQ; DQ008829
Hilu et al. 2003; Borsch 3407
BONN AF542581 (KH)
S. Kim et al., unpublished data;
AF389243

Buxaceae:
Buxus sempervirens L.
Pachysandra procumbens Michx.
Pachysandra terminalis Sieb. & Zucc.
Parkinson et al. 1999; AF193996
Chase 207, NCU; JL/LL/YQ; DQ008684
Chase 207, NCU; OD/FBQ/YQ; DQ008742
Hilu et al. 2003; Borsch 3407
BONN AF542581 (KH)
Les et al. 1999; AF092973
Qiu 9031; LL/YQ; DQ008661

Buxus sp.
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Les et al. 1999; AF108719
Qiu 9031; OD/YQ; AF479239

Cabombaceae:
Brasenia schreberi J. Gmelin
Parkinson et al. 1999; AF193982;
Qiu 91031; OD/FBQ/YQ; DQ008830
Soltis et al. 2003; AF479239

Cabomba sp.
Pachysandra procumbens Michx.
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Qiu 9031; OD/YQ; AF479239

Cabomba caroliniana A. Gray
Les et al. 1999; AF108719

Calycanthaceae:
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Parkinson et al. 1999; AF193989
Qiu 94155; OD/FBQ/YQ; DQ008780
K.W. Hilu, K. Muller, and T. Borsch, unpublished manuscipt; Borsch 3455
BONN AF543730 (KH)
Zanis et al. 2003; AY095454

Calycanthus occidentalis Hook. & Arn.
Qiu 9; JL/LL/YQ; DQ008686
Qiu 9; OD/FBQ/YQ; DQ008781
Hilu et al. 2003; Borsch 3396
BONN AF542569 (TB)
Qiu 9; OD/YQ; DQ008632

Chimonanthus praecox (L.) Link
Zanoni & Jimenez 47067; JL/LL/YQ; DQ008688
Zanoni & Jimenez 47067; OD/FBQ/YQ; DQ008805
Zanoni & Jimenez 47067; Kew; AJ966793
Zanoni & Jimenez 47067; MZ/DES/PSS AY095458

Canellaceae:
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Qiu 90017; OD/FBQ/YQ; DQ008804
K.W. Hilu, K. Muller, and T. Borsch, unpublished manuscipt; Borsch 3466
BONN AF543731 (TB)
Zanis et al. 2003; AY095455

Cinnamodendron ekmanii Sleum.
Zanoni & Jimenez 47067; JL/LL/YQ; DQ008688
Zanoni & Jimenez 47067; OD/FBQ/YQ; DQ008805
Zanoni & Jimenez 47067; Kew; AJ966793
Zanoni & Jimenez 47067; MZ/DES/PSS AY095458

Cinnamomum verum Jacq.
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<td><em>Ascarina rubricaulis</em> Solms</td>
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<td>K. Wurdack 92-0010, NCU; JL/L/L/YQ; DQ008692</td>
<td>K. Wurdack 92-0010, NCU; OD/FBQ/YQ; DQ008819</td>
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<td><em>Sarcandra chloranthaoides</em> Gardner</td>
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Ginkgoaceae:
Ginkgo biloba L. Chaw et al. 2000; AB029355 Qiu 94015; OD/FBQ/YQ; DQ008838 Hilu et al. 2003; Borsch 3469 BONN AF543736 (KH) Qiu 94015; OD/YQ; DQ008665

Gnetaceae:
Gnetum gnemon L. Qiu 97141; JL/LL/YQ; DQ008701 Qiu 97141; OD/FBQ/YQ; DQ008833 Hilu et al. 2003; Borsch 3470 BONN AF542561 (KH) Kuzoff et al. 1998; AF036488

Gyrocarpaceae:
Gyrocarpus americana Jacq. Chase 317, NCU; JL/LL/YQ; DQ008702 Chase 317, NCU; OD/FBQ/YQ; DQ008770 Chase 317, NCU; OD/YQ; DQ008624

Hernandiaceae
Hernandia nymphaefolia (Presl) Kub. Zanis et al. 2003; AY095462

Himantandraceae:
Galbulimima belgraveana (F. Muell.) Sprague Weston 929, NSW; JL/LL/YQ; DQ008704 Weston 929, NSW; OD/FBQ/YQ; DQ008788 Weston 929, NSW; Kew; AF465294 Zanis et al. 2003; AY095459

Idiospermaceae:
Idiospermum australiense (Diels) S.T. Blake Qiu 91042; JL/LL/YQ; DQ008705 Qiu 91042; OD/FBQ/YQ; DQ008782 Qiu 91042; OD/YQ; DQ008633

Illiciaceae:
Illicium floridanum Ellis Qiu 61; JL/LL/YQ; DQ008706 Qiu 61; OD/FBQ/YQ; DQ008825 K.W. Hilu, K. Müller, and T. Borsch, unpublished manuscript; Borsch 3552, BONN AF543738 (TB) Zanis et al. 2003; AY095462

Juncaginaceae:
Triglochin maritima L. Qiu 97106; JL/LL/YQ; DQ008707 Qiu 97106; OD/FBQ/YQ; DQ008811 Hilu et al. 2003 Borsch 3392 BONN AF542566 (TB) Qiu 97106; LL/YQ; DQ008650

Lactoridaceae:
Lactoris fernandeziana Phil. Chase 1014, K; JL/LL/YQ; DQ008708 Chase 1014, K; OD/FBQ/YQ; DQ008798 L.W. Chatrou et al., unpublished data; AF465297 Zanis et al. 2003; AY095463

Lardizabalaceae:
Akebia quinata Decne. Qiu 91020; JL/LL/YQ; DQ008709 Qiu 91020; OD/FBQ/YQ; DQ008761 Qiu 91020; OD/YQ; DQ008619 Hilu et al. 2003; Borsch 3412 BONN AF542587 (TB) Qiu 97135; TB/KH; AY437809 Qiu 97135; OD/YQ; DQ008618

Lauraceae:
Cinnamomum camphora (L.) T. Nees & Eberm. Qiu 102; JL/LL/YQ; DQ008711 Qiu 102; OD/FBQ/YQ; DQ008772 Qiu 102; OD/YQ; DQ008625 Qiu 102; Kew; AJ966800 Rohwer 2000; AJ247158 Qiu 98048; OD/YQ; DQ008627

Cryptocarya alba (Molina) Looser Qiu 98048; JL/LL/YQ; DQ008712 Qiu 98048; OD/FBQ/YQ; DQ008774 Qiu 98048; OD/YQ; DQ008627

Cryptocarya meisneriana Frodin Parkinson et al. 1999; AF193990 Qiu 94209; OD/FBQ/YQ; DQ008773 Qiu 94209; OD/YQ; DQ008626

Laurus nobilis L. Parkinson et al. 1999; AF193993 Qiu 28; OD/FBQ/YQ; DQ008786 Zanis et al. 2003; AY095464

Magnoliaceae:
Liriodendron chinense (Hemsl.) Sarg. Parkinson et al. 1999; AF193990 Qiu 28; OD/FBQ/YQ; DQ008786 Azuma et al. 1999; AB021016 Zanis et al. 2003; AY095464

Liriodendron tulipifera L. Magnolia denudata Desr. Magnolia grandiflora L. Chaw et al. 2000; AF161089 Zanis et al. 2003; AF389256
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<td>Nelumbo lutea (Willld.) Pers.</td>
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Zanis et al. 2003; AF389259

K.W. Hilu, K. Müller, and T. Borsch, unpublished manuscript; Borsch & Summers 3220,
FR AF543740 (TB)

Zanis et al. 2003; AF389259

Hilu et al. 2003; Borsch 3337, FR AF543741 (TB)

Zanis et al. 2003; AF389259
Pinus wallichiana A. B. Jackson

Piperaceae:

*Peperomia graveolens* Rauh & Barthlott

*Peperomia obtusifolia* A. Dietr.

*Piper betle* L.

*Piper crocatum* R. & P.

Platanaceae:

*Platanus occidentalis* L.

Podocarpaceae:

*Podocarpus costalis* Presl

*Podocarpus macrophyllus* (Thunb.) Sweet

Potamogetonaceae:

*Potamogeton berchtoldii* Fieber

*Potamogeton distinctus* Arth. Benn.

Proteaceae:

*Grevillea banksii* R. Br.

*Grevillea robusta* Cunn. & R. Br.

*Persoonia katerae* P. Weston & L. Johnson

*Persoonia katerae* P. Weston & L. Johnson

*Sabia canescens* Cunn. ex R. Br.

Ranunculaceae:

*Ranunculus* sp.

*Ranunculus ficaria* L.

*Ranunculus keniensis* Milne-Redhead & Turrill

*Xanthorhiza simplicissima* Marshall

Sabiaceae:

*Sabia* sp.

*Sabia swinhoei* Hemsl.

*Meliosma squamulata* Hance

*Meliosma veitchiorum* Hemsl.

Sargentodoxaceae:

*Sargentodoxa cuneata* (Oliv.) Rehder & Wilson
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<td>Gadek et al. 2000; AF152203</td>
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<td><em>Trochodendron aralioides</em> Sieb. &amp; Zucc. Qiu 49; JL/LL/YQ; DQ008738</td>
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Welwitschiaceae:
*Welwitschia mirabilis* Hook. f.

Chaw et al. 2000; AF161083  
Qiu M44; OD/FBQ/YQ; DQ008834  
Hilu et al. 2003; Borsch 3410, BONN AF542562 (TB)  
Qiu M44; OD/YQ; DQ008662

Winteraceae:
*Drimys winteri* J.R. & G. Forster

Parkinson et al. 1999; AF197162  
Qiu 90016; OD/FBQ/YQ; DQ008801  
Borsch 3479, BONN; TB  
Kuzoff et al. 1998; AF036491

*Takhtajania perrieri* M. Baranova & J. Leroy  
Rabenantoandro 219, MO; JL/LL/YQ; DQ008740  
Rabenantoandro 219, MO; OD/FBQ/YQ; DQ008803  
Rabenantoandro 219, MO; Kew; AJ581455  
Rabenantoandro 219, MO; OD/YQ; DQ008645  
Kuzoff et al. 2003; AY095469

*Zasmannia insipida* DC.

Qiu 90032; JL/LL/YQ; DQ008739  
Qiu 90032; OD/FBQ/YQ; DQ008802  
Qiu 90032; Kew; AJ66810  
Qiu 90032; OD/YQ; DQ008645  
Kuzoff et al. 2003; AY095469

Zamiaceae:

*Zamia floridana* A. DC.

Chaw et al. 2000; AF029357  
Qiu 95035; OD/FBQ/YQ; DQ008839  
Qiu 95035; OD/YQ; DQ008666  
S. Zhang et al., unpublished data; AF410170

*Tasmannia furfuracea* Aiton

Note. Vouchers with numbers between Qiu 1 and Qiu 93999 are deposited in NCU, Qiu 94001–Qiu 97999 in IND, Qiu 98001–Qiu 99999 in Z, and Qiu 00001–Qiu 02999 in MICH. Vouchers by collectors other than Qiu are indicated with the herbaria where they have been deposited. Sequence contributors: DES, Douglas E. Soltis; FBQ, Fabiana Bernasconi-Quadroni; KH, Khidir Hilu; JL, Jungho Lee; LL, Libo Li; MZ, Michael Zanis; OD, Olena Dombrovska; PSS, Pamela S. Soltis; TB, Thomas Borsch; YQ, Yin-Long Qiu. Numbers labeled with asterisks are DNA numbers (no voucher or a voucher by someone without a number).
Table 2
Information on New Sequences and Replacements for the Five Genes Used by Qiu et al. (2000) and Correction of Errors in Table A1 of Qiu et al. (2000)

<table>
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<th>Family and species</th>
<th>mt-atp1</th>
<th>mt-matR</th>
<th>cp-atpB</th>
<th>cp-rbcL</th>
<th>nu-18S rDNA</th>
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Hedyosmum arborescens Sw.

Cycadaceae:
Cycas revoluta Thunb.

Degeneriaceae:
Degeneria vitensis

Didymelaceae:
Didymeles perrieri Olivier

Dioscoreaceae

Eupomatiaceae:
Eupomatia bennettii F. Muell.

Eupteleaceae:
Euptelea polyandra Sieb. & Zucc.

Ginkgoaceae:
Ginkgo biloba L.

Gnetaceae:
Gnetum gnemon L.

Gomortegaceae:
Gomortega keule (Molina) L.M. Johnson

Gyrocarpaceae:
Gyrocarpus americana Jacq.

Hernandiaceae:
Hernandia ovigera L.

Himantandraceae:
Galbulimima belgraveana (F. Muell.) Sprague

Juncaginaceae:
Triglochin maritima L.

Lauraceae:
Cryptocarya obovata

Monimiaceae:
Peumus boldus Molina

Savolainen et al. 2000; AJ235491
Pryer et al. 2001; AF313558
Savolainen et al. 2000; AJ235451
Qiu et al. 1993; L12643
Soltsis et al. 2000; AF206898
Hoot et al. 1999; AF094541
Caddick et al. 2002; AF308014
Soltis et al. 1997; AF469771
Soltis et al. 1997; L75831
Graham and Olmstead 2000b; AF187060
Soltis et al. 2000; AF209593
Ueda et al. 1997; AF206773
Qiu et al. 1993; L12647
Soltsis et al. 2000; AF206923
Les et al. 1997; U80714
P. G. Martin and J. Dowd, unpublished data; L28950
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Note. New sequences are given in boldface.
that are not present in the strict consensus of the two trees indicated by asterisks.

Because the tree topologies from the two parsimony searches are generally congruent, we describe them together. Amborella, Nymphaeaceae, and Austrobaileyales form successive sister lineages to the rest of the angiosperms, with generally strong bootstrap support (we regard bootstrap values of 50%-69% as weak, 70%-84% as moderate, and 85% and above as strong support; these cutoff values are designated for convenience of communication, but see Hillis and Bull 1993 for a discussion of phylogenetic implication of bootstrap values). However, the placement of Amborella as the sister to all other angiosperms is only weakly to moderately supported. Further, five strongly supported clades are recognized within the remaining angiosperms in the five-protein-gene analysis: monocots, Chloranthaceae, Ceratophyllum, magnoliids, and eudicots. In contrast, the monophyly of magnoliids did not receive support of >50% in the nine-gene analysis. Ceratophyllum was moderately supported as the sister to eudicots in the five-protein-gene analysis but strongly supported as the sister to monocots in the nine-gene analysis. No other higher-level relationships among the basal angiosperms received bootstrap support above 50%. Finally, within the magnoliids, the sister relationships between Magnoliidae and Laurales, between Canellales and Piperales, and between these two larger clades are all strongly supported in the five-protein-gene analysis. In the nine-gene analysis, however, only the sister relationship between Magnoliidae and Laurales received strong support. The bootstrap percentages of key nodes in the trees from analyses of nine genes, five protein genes, five protein genes plus 18S rDNA, and five protein genes plus 18S and 26S rDNAs are presented in table 3.

The Bayesian analyses of the nine-gene and five-protein-gene matrices produced similar topologies, with the sole difference being that monocots and eudicots switched position as the sister to magnoliids (fig. 3). There are two additional topological features that are seen in results of the Bayesian analysis but not the parsimony analyses: Ceratophyllum is sister to Chloranthaceae (PP = 0.78 and 0.92 in the nine-gene and five-protein-gene analyses, respectively), and Amborella is sister to Nymphaeaceae (PP = 1.00 in both analyses). Otherwise, the topologies of the Bayesian and parsimony trees are similar.

The ML analyses of the nine-gene and five-protein-gene matrices also identified certain relationships that were recovered in the parsimony and Bayesian analyses, i.e., monophyly of magnoliids and placement of Amborella, Nymphaeaceae, and Austrobaileyales as successive sisters to all other extant angiosperms, but they differed in resolving relationships among Ceratophyllum, Chloranthaceae, magnoliids, monocots, and eudicots. Schematic presentations of the trees from both analyses and the bootstrap values are shown in figure 3.

The parsimony bootstrap analyses of three genome-specific matrices produced similar topologies but with different support for various relationships among basal angiosperms (fig. 4). The positions of Amborella, Nymphaeaceae, and Austrobaileyales were supported by all three genome-specific analyses, with the plastid data set giving strong support and the mitochondrial and nuclear data sets providing only moderate to weak support, respectively. Chloranthaceae, Ceratophyllum, and eudicots were each recovered with strong support in all three single-genome analyses. Monocot monophyly was strongly supported by plastid data, moderately supported by mitochondrial data, and not supported by a bootstrap value >50% by the nuclear data. The monophyly of magnoliids and relationships among the member clades (Magnoliidae, Laurales, Canellales, and Piperales) received only weak support in the plastid genome analysis. The mitochondrial and nuclear data sets contained essentially no phylogenetic signal for recognizing this clade or for resolving relationships among its subclades, with the sole exception that the sister relationship between Magnoliidae and Laurales is strongly supported by the mitochondrial data set.

In our examination of the nine-gene alignment, a total of 71 sites were identified that contain apparently synapomorphic substitutions that separate gymnosperms-Amborella-Nymphaeaceae-Austrobaileyales and all other angiosperms (fig. 5). These sites were removed, both the shortest tree search and a bootstrapping analysis of the nine-gene matrix identified Ceratophyllum as the sister to all other angiosperms, with 55% bootstrap support. Amborella, Nymphaeaceae, and Austrobaileyales formed a weakly (63%) supported clade as part of a trichotomy with monocots and a clade containing Chloranthaceae, magnoliids, and eudicots (data not shown). We also conducted a shortest tree search using the 71-site matrix (fig. 5), but because of limited information for resolving relationships among the shallow branches, the search did not finish because of the huge number of trees found and the corresponding computer memory shortage. However, in the trees recovered when the search was aborted, the angiosperms exclusive of Amborella, Nymphaeaceae, and Austrobaileyales did form a monophyletic group, with members of the latter three clades variously grouping with the gymnosperms (data not shown). These results confirm that our identification of the sites containing putatively synapomorphic substitutions was correct. The 71 sites are distributed throughout the entire length of each of the nine genes, with only 13 sites linked in five groups (fig. 5). They contain all six possible substitutional changes, with 38 sites exhibiting transitions between gymnosperms-Amborella-Nymphaeaceae-Austrobaileyales and all other

![Fig. 1](image-url) One of the six shortest trees found in the parsimony analysis of the nine-gene matrix. Numbers above branches are branch lengths (ACCTRAN optimization); those below in italics are bootstrap percentages (only those >50% are shown; for branches related to Amborella, Nymphaeaceae, Austrobaileyales, Ceratophylum, magnoliids, monocots, and eudicots, the bootstrap percentages are in boldface). The nodes labeled with asterisks are collapsed in the strict consensus of the six shortest trees. Abbreviations: GYM = gymnosperms; AMB = Amborella; NYM = Nymphaeaceae; AUS = Austrobaileyales; CHL = Chloranthaceae; CER = Ceratophyllum; MON = monocots; EUD = eudicots; CAN = Canellales; PIP = Piperales; MAG = Magnoliidae; LAU = Laurales; Acorus cal = Acorus calamus; Acorus gra = Acorus gramineus; Ceratophyllum dem = Ceratophyllum demersum; Ceratophyllum sub = Ceratophyllum submersum.
angiosperms (16 A ↔ G and 22 C ↔ T) and 33 sites showing transversions (8 A ↔ C, 8 A ↔ T, 6 C ↔ G, and 11 G ↔ T). This substitution pattern and frequency clearly contrast with what would be expected if RNA editing and GC-content bias had contributed signal to link Amborella-Nymphaeaceae-Austrobaileyales with the gymnosperms. RNA editing and reverse editing should result in far more changes of C → T, A → C, A → T, C → G, and G → T than A → G substitutions. The GC-content bias would predict many more changes of A → G, A → C, G → T, and C → T than those of A → T, and C → G. For the five protein genes, only mitochondrial atp1 has all four sites located at the third codon positions, and the other four genes (plastid atpB, matK, rbcL, and mitochondrial matR) have sites at all three codon positions, with 11, 8, and 24 sites located at the first, second, and third codon positions, respectively. For the four rDNAs, all sites are located in well-aligned conservative regions. These results indicate that the phylogenetic signal in these nine genes that supports placement of Amborella, Nymphaeaceae, and Austrobaileyales as basal lineages is not likely due to any peculiar molecular evolutionary phenomena that may cause analytical artifacts, such as RNA editing and GC-content bias.

**Discussion**

Recent molecular analyses have converged on a topology of basal angiosperm relationships in which (1) Amborella, Nymphaeaceae, and Austrobaileyales represent the basal lineages of extant angiosperms; (2) two pairs of traditional magnoliid taxa, Magnoliidae-Laurales and Canellales-Piperales, are sister to each other and form the magnoliid clade; and (3) Ceratophyllum, Chloranthaceae, monocots, magnoliids, and eudicots form a polytomy after the initial diversification that led to Amborella, Nymphaeaceae, and Austrobaileyales (Mathews and Donoghue 1999; Qiu et al. 1999, 2000; Graham and Olmstead 2000b; Soltis et al. 2000; Zanis et al. 2002, 2003; Borsch et al. 2003; Hilu et al. 2003; Löhne and Borsch 2005). This set of relationships has been used to formalize a classification system for angiosperms (APG II 2003) and to guide investigation of various aspects of early angiosperm evolution (e.g., Endress and Igersheim 2000; Friis et al. 2000; Thienn et al. 2000; Williams and Friedman 2002; Ronse De Craene et al. 2003; Fedt et al. 2004; Kramer et al. 2004). Work is still needed to establish firmly that the current consensus rests on a solid phylogenetic foundation and, more importantly, to resolve the polytomy among Ceratophyllum, Chloranthaceae, monocots, magnoliids, and eudicots. Attention to these pivotal issues in our understanding of the origin and early evolution of angiosperms is justified, especially given that three recent analyses using entire plastid genome sequences have failed to confirm that Amborella and Nymphaeae are basal lineages in angiosperm phylogeny (Goremykin et al. 2003a, 2003b, 2004) and published molecular analyses have not obtained full resolution and strong support for most higher-level relationships among basal angiosperms. Below we discuss these issues.

**Table 3**

<table>
<thead>
<tr>
<th>Clade</th>
<th>3-gene (Soltis et al. 2000) (jack knife)</th>
<th>5-gene (Qiu et al. 2000)</th>
<th>5–11 gene (Zanis et al. 2002)</th>
<th>5-protein (this study)</th>
<th>5-protein + 18S (this study)</th>
<th>5-protein + 18S + 26S (this study)</th>
<th>9-gene (this study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amborella “basal”</td>
<td>65</td>
<td>88</td>
<td>91</td>
<td>81</td>
<td>87</td>
<td>87</td>
<td>89</td>
</tr>
<tr>
<td>Amborella/Nymphaeaceae basa¹</td>
<td>72</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Amborella/Nymphaeaceae/ Austrobaileyales basal²</td>
<td>71</td>
<td>96</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>93</td>
</tr>
<tr>
<td>Magnoliids</td>
<td>&lt;50</td>
<td>62</td>
<td>78</td>
<td>86</td>
<td>77</td>
<td>65</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Laurales + Magnoliidae</td>
<td>...</td>
<td>60</td>
<td>98</td>
<td>88</td>
<td>89</td>
<td>83</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Canellales + Piperales</td>
<td>...</td>
<td>80</td>
<td>75</td>
<td>88</td>
<td>90</td>
<td>75</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Eudicots + Ceratophyllaceae</td>
<td>53</td>
<td>...</td>
<td>...</td>
<td>74</td>
<td>73</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Monocots + Ceratophyllaceae</td>
<td>...</td>
<td>&lt;50</td>
<td>57</td>
<td>...</td>
<td>...</td>
<td>50</td>
<td>88</td>
</tr>
</tbody>
</table>

Note. <50 indicates a clade that was retrieved with a data set but received bootstrap support <50%; ellipsis dots indicate a clade that was not retrieved with the data set indicated.

¹ Monophyly of all angiosperms other than Amborella.

² Monophyly of all angiosperms other than Amborella and Nymphaeaceae.

³ Monophyly of all angiosperms other than Amborella, Nymphaeaceae, and Austrobaileyales.

*Fig. 2* One of the two shortest trees found in the parsimony analysis of the five-protein-gene matrix. Numbers above branches are branch lengths (ACCTRAN optimization); those below in italics are bootstrap percentages (only those >50% are shown; for branches related to Amborella, Nymphaeaceae, Austrobaileyales, Ceratophyllum, magnoliids, monocots, and eudicots, the bootstrap percentages are in boldface). The node labeled with an asterisk is collapsed in the strict consensus of the two shortest trees. Abbreviations: GYM = gymnosperms; AMB = Amborella; NYM = Nymphaeaceae; AUS = Austrobaileyales; CHL = Chloranthaceae; CER = Ceratophyllum; MON = monocots; EUD = eudicots; CAN = Canellales; PIP = Piperales; MAG = Magnoliidae; LAU = Laurales; Acorus cal = Acorus calamus; Acorus gra = Acorus gramineus; Ceratophyllum dem = Ceratophyllum demersum; Ceratophyllum sub = Ceratophyllum submersum.
Amborella, Nymphaeaceae, and Austrobaileyales as the Basalmost Lineages of Extant Angiosperms

Several early studies hinted at the possibility that one or more of the three lineages now placed at the base of the angiosperm phylogenetic tree, *Amborella*, Nymphaeaceae, and Austrobaileyales, could represent the earliest-diverging lineages of extant angiosperms (Donoghue and Doyle 1989; Martin and Dowd 1991; Hamby and Zimmer 1992; Qiu et al. 1993; Soltis et al. 1997). However, lack of strong internal support and poor resolution in parts of the topologies prevented general acceptance of those results. In 1999–2000, several comprehensive analyses using extensive taxon and gene sampling as well as duplicate gene rooting strategy identified *Amborella*, Nymphaeaceae, and Austrobaileyales as the successive sister clades to all other angiosperms (Mathews and Donoghue 1999, 2000; Parkinson et al. 1999; Qiu et al. 1999, 2000; Soltis et al. 1999a; Barkman et al. 2000; Graham and Olmstead 2000b; Soltis et al. 2000). The impressively resolved overall topology with strong bootstrap support and a high degree of convergence of results from different research groups using different taxon and gene sampling schemes as well as different rooting strategies led to the realization that the earliest-diverging lineages of extant angiosperms had been identified. Subsequent analyses with different methods and

Fig. 3  Simplified presentation of the trees from Bayesian and fast maximum likelihood (ML) analyses of the nine-gene and five-protein-gene matrices. Taxa used in the analyses are the same as those used in figs. 1 and 2. A, Bayesian analysis of the nine-gene matrix. B, Bayesian analysis of the five-protein-gene matrix. C, ML analysis of the nine-gene matrix. D, ML analysis of the five-protein-gene matrix. The numbers above the branches are posterior probabilities for Bayesian analyses or bootstrap values for ML analyses. Abbreviations: GYM = gymnosperms; AMB = *Amborella*; NYM = Nymphaeaceae; AUS = Austrobaileyales; CHL = Chloranthaceae; CER = Ceratophyllum; MON = monocots; EUD = eudicots; CAN = Canellales; PIP = Piperales; MAG = Magnoliidae; LAU = Laurales.

from all other angiosperms and found that these changes are distributed in all nine genes from the three genomes and include all six possible substitutional changes at frequencies that do not seem to be biased by RNA editing or GC-content bias (fig. 5). This result, together with previously published tests (Qiu et al. 2000, 2001) that showed that the Amborella-Nymphaeaceae-Austrobaileyales rooting in our earlier analyses (Qiu et al. 1999) was unaffected by long branch attraction, suggests that the strategy of using multiple genes and dense “judicious” taxon sampling (Hillis 1998) is effective in tackling the recalcitrant problem of determining the earliest-diverging lineages of extant angiosperms.

In their most recent study, Goremykin et al. (2004) presented a comparison of putative synapomorphic substitutions between the Poaceae-basal or the Amborella-Nymphaeaceae-Austrobaileyales-basal topologies and found that there are more sites supporting the former than the latter. We note that their use of a single gymnosperm (Pinus) as the outgroup, use of Poaceae as the only representatives of monocots, and exclusion of the third codon positions could lead to misidentification and underdetection of synapomorphic sites. In our analysis, we applied a more stringent criterion to score a site as synapomorphic; namely, it had to be conserved in at least two of the four gymnosperm lineages and two of Amborella, Nymphaeaceae, and Austrobaileyales but with a largely invariable different nucleotide in all other angiosperms. Furthermore, conservation of the five linked sites in the mtSSU, GTGTG in gymnosperms-Austrobaileyales (fig. 5) actually extends to Adiantum, Huperzia, and Lycophodium (Duff and Nickrent 1999) and possibly throughout all nonflowering land plants (Oda et al. 1992; Duff and Nickrent 1999; Parkinson et al. 1999; Chaw et al. 2000). Moreover, 28 of 47 sites that contain synapomorphic substitutions in the five protein genes are located at the third codon positions. Thus, we argue that the sites we identified are free of the problems of insufficient taxon sampling and bias and probably represent many of the sites that contain phylogenetic signal for resolving the basalmost angiosperm issue.

Finally, the placement of Amborella, Nymphaeaceae, and Austrobaileyales as basal lineages is supported by all three single-genome analyses (fig. 4), passing the test that a robust understanding of organismal phylogeny should be supported by analysis of all genomes within the organism (Qu and Palmer 1999). Additionally, both the nine-gene and five-protein-gene analyses using parsimony, ML, and Bayesian methods give strong support to this topology. In consideration of the variety of analyses we have conducted on our multi-gene data set in this and previous studies (Qiu et al. 1999, 2000, 2001), it is safe to conclude that the Amborella-Nymphaeaceae-Austrobaileyales-basal topology of the angiosperm phylogeny has been rigorously tested. Moreover, the congruent topologies inferred from functionally and structurally different coding genes in this study and others (e.g., phytochromes: Mathews and Donoghue 1999, 2000; floral MADS-box genes: Kim et al. 2004) and noncoding DNAs in the analyses of Borsch et al. (2003) and Löhne and Borsch (2005) should make sufficiently clear that locus-inherent specific patterns of molecular evolution have not led to a spurious conclusion of the rooting of angiosperm phylogeny.
Monophyly of and Relationships within the Magnoliids

Initial support for the magnoliid clade (Qiu et al. 1999, 2000) was not strong, and morphological evidence was lacking (Doyle and Endress 2000). However, other analyses with different methods and data have consistently corroborated this finding (Mathews and Donoghue 1999; Barkman et al. 2000; Graham and Olmstead 2000b; Zanis et al. 2002, 2003; Borsch et al. 2003; Hilu et al. 2003). Recent analysis of the group II intron in petD also found a synapomorphic indel for the magnoliid clade (Löhne and Borsch 2005). The parsimony analysis of the five-protein-gene matrix in this study yielded strong bootstrap support for both monophyly of the magnoliids and relationships among the four member subclades (fig. 2). Further, Bayesian and ML analyses of both nine-gene and five-protein-gene matrices recovered this clade and resolved the same set of relationships, despite with varying PP and bootstrap values (fig. 3). Thus, it is reasonable to say that the magnoliids represent a major clade of basal angiosperms. The taxa included in this clade represent a majority of the traditional ranalian complex (Qiu et al. 1993). With Amborella, Nymphaceae, Austrobaileyales, Ceratophyllum, Chloranthaceae, Ranunculales, Papaverales, and Nelumbo removed, all other taxa of Cronquist's (1981) subclass Magnoliidae remain as magnoliids.

Identification of this large magnoliid clade significantly enhances clarification and will aid further resolution of relationships among basal angiosperms. It effectively reduces the options for placing Chloranthaceae, a family that has been placed previously with Laurales (Thorne 1992), Piperales (Cronquist 1981), and Canellales (Dahlgren 1989) and that is still uncertain for its phylogenetic affinity. Furthermore, placement of Piperales as sister to Canellales within the magnoliids removes the order from the list of taxa to be considered as potential sister lineages to monocots, as Burger (1977) suggested a close relationship between Piperales and monocots. Similarly, Magnoliidae (termed as Annonales then) alone can no longer be entertained as a potential sister group to monocots, as proposed by Dahlgren et al. (1985), since they are embedded within the magnoliid clade.

The close relationship between Magnoliidae and Laurales was clearly recognized in the premolecular systematics era (Cronquist 1981). Two genome-specific analyses (plastid and mitochondrial), the nine-gene analysis, and the five-protein-gene analysis all identified this relationship, generally with strong support (figs. 1–4). Winteraceae and Canellaceae (collectively classified as Canellales; APG II 2003), traditionally placed in Magnoliidae (Cronquist 1981) and still associated with that order in a morphological cladistic analysis by Doyle and Endress (2000), consistently appear as the sister to Piperales. The two larger clades, Magnoliidae-Laurales and Canellales-Piperales, are sister to each other in all analyses that recovered the magnoliid clade (Mathews and Donoghue 1999; Graham and Olmstead 2000b; Zanis et al. 2002, 2003; Borsch et al. 2003; Hilu et al. 2003). Hence, these relationships among the magnoliid lineages can be deemed robust. However, they are different from those depicted by a morphological cladistic analysis (Doyle and Endress 2000). Convergence at the morphological level may be a factor. Future investigations of the development of morphological characters using molecular genetic approaches (e.g., Buzgo et al. 2004; Kramer et al. 2004) and other nonmolecular characters may sort out homoplasy and identify proper synapomorphies for the several clades identified here.

Relationships among Ceratophyllum, Chloranthaceae, Monocots, Magnoliids, and Eudicots

The primary remaining challenge is to resolve relationships among Ceratophyllum, Chloranthaceae, monocots, magnoliids, and eudicots. The highly divergent nature of Ceratophyllum was noticed by Les and his colleagues as early as 1988 and 1991, based on both morphological and molecular evidence. The phylogenetic affinity of this genus remains elusive. Based on bootstrap support for the placement of Ceratophyllum, which is moderate at best, our nine-gene and five-protein-gene analyses present two alternative hypotheses on the placement of the genus, sister to monocots and eudicots, respectively (figs. 1, 2; fig. 3C, 3D). The relationship of Ceratophyllum to eudicots was reported by Soltis et al. (2000) with only 53% jackknife support, by Hilu et al. (2003) with 71% jackknife support, and by Graham et al. (forthcoming) with 82% bootstrap support. The 74% parsimony bootstrap value and 53% ML bootstrap value in our five-protein-gene analyses (fig. 2) support this relationship to eudicots. In contrast, the placement with the monocots supported by our nine-gene analysis using both parsimony and ML methods is undermined by a topological anomaly within the monocots, i.e., the sister relationship of Acorus to alismatids (fig. 1). The correct placement of Acorus is sister to all other monocots according to several analyses with a large monocot sampling (Chase et al. 2000, forthcoming; Soltis et al. 2000; Hilu et al. 2003). The erroneous position of Acorus here could indicate that the placement of Ceratophyllum in the nine-gene analysis is an artifact. Indeed, for all four mitochondrial genes we used (atp1, matR, mtSSU, and mtLSU), Ceratophyllum, Acorus, and alismatids have highly divergent sequences in comparison to other basal angiosperms, indicating that they could attract to each other as long branches. The relationship of Ceratophyllum to Chloranthaceae, shown by our Bayesian analyses of both the nine-gene

Fig. 5 “Synapomorphic substitutions” that separate gymnosperms-Amborella-Nymphaceae-Austrobaileyales (or just Amborella and Nymphaceae in some cases) from all other angiosperms in plastid atpB, matK, and rbcL, mitochondrial matR, atp1, mtLSU, and mtSSU, and nuclear 18S and 26S rDNAs. The numbers in the top row refer to codon positions in the protein genes. A hyphen indicates missing data; a tilde (−) indicates a gap; dots denote the same nucleotides as in Magnolia (the top sequence). The underlined sites are contiguous in the original alignment, and all other sites are distributed individually throughout the gene. Abbreviations: GYM = gymnosperms; AMB = Amborella; NYM = Nymphaceae; AUS = Austrobaileyales; CHL = Chloranthaceae; CER = Ceratophyllum; MON = monocots; EUD = eudicots; CAN = Canellales; PIP = Piperales; MAG = Magnoliaceae; LAU = Laurales.
Chloranthaceae instead of eudicots (fig. 3). Alternatively, the
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(2002), which involved a matrix of five to 11 genes. In this
Support for many critical relationships among basal angio-
sperm relationships as sequences from 18S rDNA and
support for angiosperm relationships (including basal angio-
sperms (to 59%), with support for the monophyly of magno-
lids and also of Canellales þ Piperales both decreased compared to the five-protein-gene analysis. The placement of Ceratophyllum also changed with the addition of 26S (table 3).

The most dramatic change in the internal support for clades resulted from the addition of the two mitochondrial rDNAs. The addition of these two genes resulted in a sharp drop in the support for Amborella as sister to all other angiosperms (to 59%), with support for the monophyly of magnoliids and also of Canellales þ Piperales dropping below 50%. These two mitochondrial genes appear to be adding conflicting signal to that from the protein-coding and nuclear 18S rDNA. Conflict is also evident among data sets regarding the placement of Ceratophyllum as sister to either eudicots or monocots (table 3). The conflict introduced by mtSSU and mtLSU with regard to monophyly of magnoliids and relationships among their member clades seems to be caused by lineage-specific rate heterogeneity in these two genes (data not shown), whereas the drop in support for Amborella as the sister to all other angiosperms after addition of these two genes reflects a genuine uncertainty on the exact topology at the first node in the angiosperm phylogeny, as Amborella and Nymphaeaceae together are supported as the earliest-diverging lineage in three of the six analyses performed in this study (fig. 3; Barkman et al. 2000; Qiu et al. 2000; Stefanovic et al. 2004). More data are clearly needed to resolve this kind of conflict among different genes.

The comparisons we have conducted (table 3) provide a valuable lesson in the addition of genes. Although total evidence is a preferred approach (Soltis et al. 1998, 2000; Qiu et al. 1999; Savolainen et al. 2000), with some investigators advocating the combination of many genes (Rokas et al. 2003), it is important to stress that not all genes contain the same amount of information for phylogenetic reconstruction (Hilu et al. 2003) and that not all genes have the same history (Maddison 1997). These gene-specific effects are caused by differences in size and internal mutational dynamics and have to be considered in addition to well-known effects of different evolutionary histories caused by reticulations or

Our analyses, as well as several earlier studies of the angio-
sperm phylogeny, revealed a steady increase in resolution and internal support for relationships as genes were added to ini-
tial single-gene matrices to form multigene data sets. For ex-
ample, Soltis et al. (1998) revealed a steady increase in support for angiosperm relationships (including basal angio-
sperm relationships) as sequences from 18S rDNA and atpB
were added to an rbcL data matrix to form two and three-
gene data sets (also Soltis et al. 1999a, 2000; Savolainen et al. 2000; table 3). Similarly, Qiu et al. (1999, 2000) also observed an increase in the support for basal angiosperm relationships in an analysis of a five-gene data set (table 3). Support for many critical relationships among basal angiosperms continued to increase in the analyses of Zanis et al. (2002), which involved a matrix of five to 11 genes. In this study, phylogenetic analysis of the five protein-coding genes (atpB, matK, rbcL, atp1, and matR) yielded a topology and internal support for relationships generally comparable to those realized in the earlier multigene analysis of Zanis et al. (2002), with the exception of Ceratophyllum, which was placed differently in the two studies. Much of the increase in internal support from these five protein-coding genes com-
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vided by matK. In fact, the rapidly evolving matK alone pro-
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(Hilu et al. 2003). Our analyses indicate that the addition of the two nuclear rDNAs does not increase the support for most of the critical nodes we examined (table 3). For exam-
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Conclusions

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lineage sorting. Although total evidence is encouraged, it is important to evaluate the contribution and impact of individual genes. In our analyses, for example, the addition of two mitochondrial and nuclear 26S rDNAs had a negative impact on resolution and support for certain parts of the tree. Chase et al. (forthcoming) also observed that in a seven-gene combined analysis of monocots, the addition of 18S and partial 26S did not increase support and for some clades resulted in weaker support than a combined analysis of protein-coding plastid genes. Therefore, the total evidence approach needs to be taken with caution.

Besides amassing multigene sequence data for a large number of taxa, a different approach also promises to resolve the relationships among major angiosperm lineages, i.e., to search for informative genomic structural changes such as those reported for resolving the origin of and relationships within land plants (Manhart and Palmer 1990; Raubeson and Jansen 1992; Qiu et al. 1998; Lee and Manhart 2002; Dombrovskia and Qiu 2003; Qiu and Palmer 2003; Quandt et al. 2004; Löhne and Borsch 2005). This approach is especially promising given that the entire plastid genome from an increasing number of angiosperms and other land plants has been sequenced (Goremykin et al. 2003a, 2003b; 2004), and more work is in progress. However, caution must be taken to ensure an appropriate taxonomic coverage so that homoplasmous changes can be distinguished from homoplasious ones (Qiu and Palmer 2004).

Therefore, we recommend that future efforts be directed toward exploration of more data, for both sequences and gene/genome structural features, with proper attention paid to both quality and quantity of taxon and character sampling. The most effective and efficient ways to analyze the resulting large matrices remain parsimony methods, which have been shown to be robust even when data are heterogeneous (Kolaczkowski and Thornton 2004). Bayesian bootstrapping (Douady et al. 2003), when it can be practically implemented, will also be worth pursuing on these large matrices. The fast ML method developed by Guindon and Gascuel (2003) provides a third possibility for analyzing large data matrices as demonstrated in this study. Careful evaluation of support values using bootstrapping or jackknifing (internal support, Nei et al. 1998) as well as congruence with other evidence (external support, Chase et al. 1993; taxonomic congruence, Miyamoto and Fitch 1995) will be essential to ensure correct interpretation of analytical results.

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