Phylogenetic Analyses of Basal Angiosperms Based on Nine Plastid, Mitochondrial, and Nuclear Genes

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PHYLOGENETIC ANALYSES OF BASAL ANGIOSPERMS BASED ON NINE PLASTID, MITOCHONDRIAL, AND NUCLEAR GENES

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Vincent Savolainen,|| Lars W. Chatrou,## and Mark W. Chase||

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DNA sequences of nine genes (plastid: atpB, matK, and rbcl; mitochondrial: atp1, matR, mtSSU, and mtLSU; nuclear: 18S and 26S rDNAs) from 100 species of basal angiosperms and gymnosperms were analyzed using parsimony, Bayesian, and maximum likelihood methods. All of these analyses support the following consensus of relationships among basal angiosperms. First, Amborella, Nymphaeaceae, and Austrobaileyales are strongly supported as a basal grade in the angiosperm phylogeny, with either Amborella or Amborella and Nymphaeales as sister to all other angiosperms. An examination of nucleotide substitution patterns of all nine genes ruled out any possibility of analytical artifacts because of RNA editing and GC-content bias in placing these taxa at the base of the angiosperm phylogeny. Second, Magnoliidae are sister to Laurales and Piperales are sister to Ceanellales. These four orders together constitute the magnoliid clade. Finally, the relationships among Ceratophyllum, Chloranthaceae, monocots, magnoliids, and eudicots are resolved in different ways in various analyses, mostly with low support. Our study indicates caution in total evidence approaches in that some of the genes employed (e.g., mtSSU, mtLSU, and nuclear 26S rDNA) added signal that conflicted with the other genes in resolving certain parts of the phylogenetic tree.

Keywords: basal angiosperms, Amborella, magnoliids, multigene analysis, synapomorphic substitutions, phylogeny.

Introduction

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 artificial 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 basal-most angiosperms (Goremykin et al. 2003a, 2003b, 2004; but see Soltis and Soltis 1998; Soltis et al. 2000; 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, Gomortegaceae (Renes 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; Berghthorsson 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 matK), Hortonia (matK), 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. Dombrovskova 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 extant angiosperms is supported by data from the plastid, mitochondrial, and nuclear genomes separately. This type of analysis has only been conducted occasionally (Mathews and
analyses, 1000 resampling replicates were performed (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 (Solits et al. 2004). Some poorly understood molecular evolutionary phenomena, such as RNA editing (Steinhauser et al. 1999; Kugita et al. 2003; Dombrovski 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 (Rodrı´ guez 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>
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<th>mt-LSU rDNA</th>
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<th>nu-26S rDNA</th>
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<td>K.W. Hilu, 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

*Daphnandra micrantha* (Tul.) Benth.
- Whalen 132, NSW; JL/LL/YQ; DQ008680
- Whalen 132, NSW; OD/FBQ/YQ; DQ008776
- Hiu et al. 2003; Borsch 3409; BONN AF542568 (TB)
- Qiu 98109; OD/YQ; DQ008630

Doryphora sassafras Endl.
- Qiu 98109; JL/LL/YQ; DQ008681
- Qiu 98109; OD/FBQ/YQ; DQ008777
- Hiu et al. 2003; Borsch 3464; BONN AF543726 (TB)
- Zanis et al. 2003; AY095452

Austrobaileyaceae:
*Austrobaileya scandens* C. T. White
- Parkinson et al. 1999; AF193988
- Qiu 90030; OD/FBQ/YQ; DQ008827
- Hilu et al. 2003; Borsch 3464; BONN AF543726 (TB)
- Zanis et al. 2003; AY095452

Berberidaceae:
*Mahonia bealei* (Fortune) Carr.
- Qiu 74; JL/LL/YQ; DQ008682
- Qiu 74; OD/FBQ/YQ; DQ008754
- Hilu et al. 2003; Borsch 3405; BONN AF542585 (TB)
- Qiu 90031; OD/YQ; DQ008613

*Mahonia japonica*
- Qiu 92003; JL/LL/YQ; DQ008683
- Qiu 92003; OD/FBQ/YQ; DQ008753
- Hilu et al. 2003; Borsch 3393; BONN AF542586 (TB)
- Qiu 92003; OD/YQ; DQ008614

*Buxus sempervirens* L.
- Qiu 97057; OD/FBQ/YQ; DQ008743
- Qiu 97057; Kew; AJ966792
- S. Kim et al., unpublished data; AF389243
- Qiu 99031; LL/YQ; DQ008661

Buxaceae:
*Buxus sp.*
- 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)
- Qiu 90031; OD/YQ; DQ008661

*Pachysandra procumbens* Michx.
- Qiu 91031; JL/LL/YQ; DQ008685
- Qiu 91031; OD/FBQ/YQ; DQ008830
- Les et al. 1999; AF092973
- Qiu 91031; LL/YQ; DQ008661

*Pachysandra terminalis* Sieb. & Zucc.
- Qiu 97027; OD/FBQ/YQ; DQ008831
- Qiu 97027; OD/FBQ/YQ; DQ008831
- Les et al. 1999; AF108719
- Solan et al. 2003; AF479239

Cabombaceae:
*Brasenia schreberi* J. Gmelin
- Qiu 91031; JL/LL/YQ; DQ008685
- Qiu 91031; OD/FBQ/YQ; DQ008830
- Les et al. 1999; AF092973
- Qiu 91031; LL/YQ; DQ008661

*Cabomba* sp.
- Parkinson et al. 1999; AF193982;
- Qiu 94155; OD/FBQ/YQ; DQ008780
- K.W. Hilu, K. Muller, and T. Borsch, unpublished manuscript; Borsch 3455; BONN AF543730 (KH)
- Zanis et al. 2003; AY095454

*Cabomba caroliniana* A. Gray
- Qiu 91031; JL/LL/YQ; DQ008685
- Qiu 91031; OD/FBQ/YQ; DQ008830
- Les et al. 1999; AF108719
- Solan et al. 2003; AF479239

Calycanthaceae:
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- Qiu 91031; OD/FBQ/YQ; DQ008830
- Les et al. 1999; AF092973
- Qiu 91031; LL/YQ; DQ008661

*Calycanthus occidentalis* Hook. & Arn.
- Qiu 91031; JL/LL/YQ; DQ008685
- Qiu 91031; OD/FBQ/YQ; DQ008830
- Les et al. 1999; AF108719
- Solan et al. 2003; AF479239

*Chimonanthus praecox* (L.) Link
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- Qiu 9; OD/FBQ/YQ; DQ008781
- Hilu et al. 2003; Borsch 3396; BONN AF542569 (TB)
- Zanis et al. 2003; AY095454

Canellaceae:
*Canella winterana* (L.) Gaertn.
- Qiu 90017; JL/LL/YQ; DQ008687
- Qiu 90017; OD/FBQ/YQ; DQ008804
- K.W. Hilu, K. Muller, and T. Borsch, unpublished manuscript; 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
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- Zanoni & Jimenez 47067; MZ/DES/SS AY095458
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<td>Parks sn, IND, Qiu 95003*; OD/FBQ/YQ; DQ008766</td>
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<td>Thien 500, NO; OD/FBQ/YQ; DQ008821</td>
<td>Thien 500, NO Kew; AJ966795</td>
<td>Hilu et al. 2003; Borsch 3467 BONN AF543733 (KH)</td>
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<td><em>Chloranthus brachystachys</em> Bl.</td>
<td>K. Wurdack 92-0010, NCU; JL/LL/YQ; DQ008692</td>
<td>K. Wurdack 92-0010, NCU; OD/FBQ/YQ; DQ008819</td>
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<td><em>Hedyosmum bonplandianum</em> L.</td>
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<td>Zanis et al. 2003; AY095461 Qiu 92002; OD/YQ; DQ008655</td>
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<td>Chaw et al. 2000; AB029356</td>
<td>Qiu 94051; OD/FBQ/YQ; DQ008840</td>
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<td>Wang et al. 2000; AF143440 Qiu 97021; OD/YQ; DQ008648</td>
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<td><em>Cycas panzhihuensis</em></td>
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<td><em>Cardiiodocia palmata</em> Ruiz &amp; Pavon</td>
<td>Qiu 97021; JL/LL/YQ; DQ008694</td>
<td>Qiu 97021; OD/FBQ/YQ; DQ008809</td>
<td>Hilu et al. 2003; Roth sn, BONN AF542578 (TB)</td>
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<td>JM Miller 1189-63; OD/FBQ/YQ; DQ008787</td>
<td>Azuma et al., unpublished data; AB053549</td>
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<td>Qiu 90022; OD/FBQ/YQ; DQ008785</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 nymphaeifolia (Presl) Kub. Zanis et al. 2003; AY095462

Hernandia ovigera L. Qiu 01007; JL/LL/YQ; DQ008703 Qiu 01007; OD/FBQ/YQ; DQ008771 Qiu 01007; Kew; AJ966799 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; AJ465294 Zanis et al. 2003; AY095459

Idiospermaceae:
Idiospermum australiense (Diels) S.T. Blake Qiu 91042; JL/LL/YQ; DQ008705 Qiu 91042; OD/FBQ/YQ; DQ008825 K.W. Hilu, K. Müller, and T. Borsch, unpublished manuscript; Borsch 3552, BONN AF543738 (TB) Zanis et al. 2003; AY095463

Illiciaceae:
Illicium floridanum Ellis Qiu 61; JL/LL/YQ; DQ008706 Qiu 61; OD/FBQ/YQ; DQ008825 Qiu 94209; OD/YQ; DQ008626 Qiu 94209; Kew; AJ966800 Qiu 94209; OD/YQ; DQ008659

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 Zanis et al. 2003; AY095463

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 Dece. Qiu 91020; JL/LL/YQ; DQ008709 Qiu 91020; OD/FBQ/YQ; DQ008761 Hilu et al. 2003; Borsch 3412 BONN AF542587 (TB) Qiu 91020; OD/YQ; DQ008619 K.W. Hilu, K. Müller, and T. Borsch, unpublished manuscript; Borsch 3552, BONN AF543738 (TB) Qiu 91020; OD/YQ; DQ008618

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

Cryptocarya alba (Molina) Looser Qiu 98048; JL/LL/YQ; DQ008712 Qiu 98048; OD/FBQ/YQ; DQ008774 Qiu 98048; OD/YQ; DQ008627 Qiu 98048; OD/YQ; DQ008624

Cryptocarya meisneriana Frodin Parkinson et al. 1999; AF193990 Qiu 94209; OD/FBQ/YQ; DQ008773 Qiu 94209; Kew; AJ966801 Qiu 94209; OD/YQ; DQ008626 Zanis et al. 2003; AY095464

Laurus nobilis L. Parkison et al. 1999; AF193990 Qiu 28; OD/FBQ/YQ; DQ008786 Azuma et al. 1999; AB021016 Zanis et al. 2003; AY095464

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

Liriodendron tulipifera L. Magnolia demudata Desr. Magnolia grandiflora L. Chaw et al. 2000; AF161089
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<td><em>Pinus douglasiana</em> Martinez</td>
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Table 1
(Continued)
**Pinus wallichiana** A. B. Jackson

**Piperaceae:**

*Peperomia graveolens* Rauh & Barthlott

Peperomia obtusifolia A. Dietr. Qiu 94135; OD/FBQ/YQ; DQ008837

*Piper betle* L. Chaw et al. 2000; AF161090

*Piper crocatum* R. & P. Hilu et al. 2003; Slotta s.n., VPI AF543745 (KH)

**Platanaceae:**

*Platanus occidentalis* L. Chaw et al. 2000; AF161090

**Podocarpaceae:**

*Podocarpus costalis* Presl

*Podocarpus macrophyllus* (Thunb.) Sweet Qiu 95006; OD/FBQ/YQ; DQ008837

**Potamogetonaceae:**

*Potamogeton berchtoldii* Fieber

*Potamogeton distinctus* Arth. Benn. Tanaka et al. 1997; AB002581

**Proteaceae:**

*Grevillea banksii* R. Br. Hilu et al. 2003; Borsch 3413 BONN AF542583 (TB)

*Grevillea robusta* Cunn. & R. Br. Parkinson et al. 1999; AF193995

*Persoonia katerae* P. Weston & L. Johnson Qiu 95006; OD/FBQ/YQ; DQ008837

**Ranunculaceae:**

*Ranunculus sp.* Chaw et al. 2000; AF161093

*Ranunculus ficaria* L. Borsch 3554 BONN; TB; AY437814

*Ranunculus keniensis* Milne-Redhead & Turrill Zanis et al. 2003; AF389269

*Xanthorhiza simplicissima* Marshall Qiu 91030; OD/FBQ/YQ; DQ008837

**Sabiaceae:**

*Sabiesa* sp. Qiu 91025; OD/FBQ/YQ; DQ008837

*Sabia swinhoei* Hemsl. Zanis et al. 2003; AF389272

*Meliosma squamulata* Hance B. Shih 3749, HAST; JL/LL/YQ; DQ008837

**Sargentodoxaceae:**

*Meliosma veitchii* Hemsl. Chase 2989, K; Kew; AJ966807

*Sargentodoxa cuneata* (Oliv.) Rehder & Wilson Pan 93001, NCU; OD/YQ; DQ008837
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<td>Houttuynia cordata Thunb.</td>
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<td>Qiu 92016; OD/FBQ/YQ; DQ008793</td>
<td>K.W. Hilu, K. Müller, and T. Borsch, unpublished manuscript; Borsch &amp; Wilde 3108, VPI &amp; FR AF543749 (TB)</td>
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<td><em>Kadsura japonica</em> (L.) Dunal Parkinson et al. 1999</td>
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<td>Parkinson et al. 1999; AF193985</td>
<td>Parks sn, IND, Qiu 94165*; OD/FBQ/YQ; DQ008824</td>
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<td><em>Siparuna decipiens</em> (Tul.) A. DC.</td>
<td>Sothers 911, MO; JL/LL/YQ; DQ008733</td>
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<td><em>Metasequoia glyptostroboides</em> Hu &amp; Cheng</td>
<td>Qiu 95084; OD/FBQ/YQ; DQ008836</td>
<td>Gadek et al. 2000; AF152203</td>
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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 Dombrovksa; 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).
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*Hedyosmum arborescens* Sw.

**Cycadaceae:**
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**Degeneriaceae:**
- *Degeneria vitensis* JM Miller 1189-63; JL/FBQ/YQ; AF293752
  - Savolainen et al. 2000; AJ235491
- *Degeneria perrieri* Olivier
  - Pryer et al. 2001; AF313558

**Didymelaceae:**
- *Didymeles perrieri* Olivier

**Dioscoreaceae**
- Caddick et al. 2002; AF308014

**Eupomatiaceae:**
- *Eupomatia bennettii* F. Muell.

**Eupteleaceae:**
- *Euptelea polyandra* Sieb. & Zucc.

**Ginkgoaceae:**
- *Ginkgo biloba* L.

**Gnetaceae:**
- *Gnetum gnemon* L. Graham and Olmstead 2000b; AF187060

**Gomortegaceae:**
- *Gomortega keule* (Molina) I.M. Johnson

**Gyrocarpaceae:**
- *Gyrocarpus americana* Jacq.
  - Chase 317, NCU; JL/FBQ/YQ; AF197701
  - Savolainen et al. 2000; AJ235481
  - Ueda et al. 1997; AF206773

**Hernandiaceae:**
- *Hernandia ovigera* L.
  - Qiu 96255*; JL/YQ; DQ007413
  - Soltsis et al. 2000; AF209593

**Himantandraceae:**
- *Galbulimima belgraveana* (F. Muell.) Sprague
  - Les et al. 1997; U80714

**Juncaginaceae:**
- *Triglochin maritima* L.

**Lauraceae:**
- *Cryptocarya obovata* P. G. Martin and J. Dowd, unpublished data; L28950

**Monimiaceae:**
- *Peumus boldus* Molina
  - Soltsis et al. 2000; AF206807
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Tofieldiaceae:
Tofeia tenufolia Michaux

Trimeniaceae:
Trimenia moorei W.R. Philipson
ANBG 701680; JL/
YQ;DQ007415

Welwitschiaceae:
Welwitschia mirabilis Hook. f.
S.W. Graham et al.,
unpublished data;
AF239795

Winteraceae:
Takhtajania perrieri M. Baranova & J. Leroy
Rabenantoandro 219,
MO; LL/YQ;
DQ007416
Rabenantoandro 219,
MO; LL/YQ;
DQ007427
Soltis et al. 2000;
AF209683

Tasmannia insipida
Hoot et al. 1999;
AF093424

Zamiaceae:
Zamia furfuracea Aiton
Graham and Olmstead
2000a; AF188845

Zamia pumila L.
Nairn and Ferl 1988;
M20017

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 implications 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 Magnoliaceae 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 Magnoliaceae 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 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 on 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 all 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 (Magnoliaceae, 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 Magnoliaceae 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). With these sites 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

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**Fig. 1** 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, *Ceratophyllum*, 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 = Magnoliaceae; LAU = Laurales; *Acorus cal* = Acorus calamus; Acorus gra = Acorus gramineus; Ceratophyllum dem = Ceratophyllum demersum; Ceratophyllum sub = Ceratophyllum submersum.
angiosperms (16 A ¼ G and 22 C ¼ T) and 33 sites showing transversions (8 A ¼ C, 8 A ¼ T, 6 C ¼ G, and 11 G ¼ 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 ¼ T, A ¼ C, A ¼ T, C ¼ G, and G ¼ T than A ¼ G substitutions. The GC-content bias would predict many more changes of A ¼ G, A ¼ C, G ¼ T, and C ¼ T than those of A ¼ T, and C ¼ 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; Thiern et al. 2000; Williams and Friedman 2002; Ronse De Craene et al. 2003; Feild 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.

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 = Magnoliaceae; LAU = Laurales; Acorus cal = Acorus calamus; Acorus gra = Acorus gramineus; Ceratophyllum dem = Ceratophyllum demersum; Ceratophyllum sub = Ceratophyllum submersum.

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 + 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>89</td>
</tr>
<tr>
<td>Amborella/Nymphaeaceae basal</td>
<td>72</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Ceratophyllaceae/Austrobaileyales basal</td>
<td>&lt;50</td>
<td>62</td>
<td>78</td>
<td>86</td>
<td>77</td>
<td>65</td>
</tr>
<tr>
<td>Magnoliids</td>
<td>...</td>
<td>...</td>
<td>98</td>
<td>88</td>
<td>89</td>
<td>93</td>
</tr>
<tr>
<td>Laurales + Magnoliidae</td>
<td>...</td>
<td>60</td>
<td>98</td>
<td>88</td>
<td>89</td>
<td>83</td>
</tr>
<tr>
<td>Canellales + Piperales</td>
<td>...</td>
<td>80</td>
<td>75</td>
<td>88</td>
<td>90</td>
<td>75</td>
</tr>
<tr>
<td>Eudicots + Ceratophyllaceae</td>
<td>53</td>
<td>...</td>
<td>...</td>
<td>74</td>
<td>73</td>
<td>...</td>
</tr>
<tr>
<td>Monocots + Ceratophyllaceae</td>
<td>...</td>
<td>&lt;50</td>
<td>57</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%; ellipses dots indicate a clade that was not retrieved with the data set indicated.

a Monophyly of all angiosperms other than Amborella.
b Monophyly of all angiosperms other than Amborella and Nymphaeaceae.
c Monophyly of all angiosperms other than Amborella, Nymphaeaceae, and Austrobaileyales.
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
new data have further confirmed and reinforced this consensus (Qiu et al. 2001; Zanis et al. 2002, 2003; Borsch et al. 2003; Hilu et al. 2003; Löhne and Borsch 2005).

In contrast to this seemingly well-established earlier consensus, three recent analyses by Goremykin et al. (2003a, 2003b, 2004) using entire plastid genome sequences failed to place Amborella and Nymphaea as basal lineages of angiosperms. Although the scanty taxon sampling, particularly of monocots, which occupy the basalmost position among angiosperms in the trees obtained by these authors, raises doubt about the validity of their conclusions (Soltis and Soltis 2004; Soltis et al. 2004; Stefanovic et al. 2004), it is important that we scrutinize our own data and analyses to ensure that our conclusions are not biased by any analytical problem. Despite theoretical understanding of several long-standing issues in phylogenetics, such as long branch attraction (Felsenstein 1978) and the trade-off between taxon versus character sampling (Hillis 1996, 1998; Graybeal 1998; Soltis et al. 1998; Zwickl and Hillis 2002), it is still not clear how best to diagnose the effects of long branch attraction or inadequate taxon or character sampling in empirical studies. We have therefore conducted various kinds of analyses since our initial publications to detect any possible “misbehavior” of the data that might have contributed to the topology we obtained (cf. Qiu et al. 2000, 2001).

In this study, we further examined the substitutions separating gymnosperms-Amborella-Nymphaeaceae-Austrobaileyales 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-Amborella-Nymphaeaceae (fig. 5) actually extends to Adiantum, Huperzia, and Lycoperdium (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 organizational phylogeny should be supported by analysis of all genomes within the organism (Qiu 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 (Qi et al. 1993).

With Amborella, Nymphaeaceae, 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, Magnoliaceae (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 Magnoliaceae 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 Magnoliaceae (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, Magnoliaceae-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 Solis 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; Solis 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
(PP = 0.78) and five-protein-gene (PP = 0.92) matrices (fig. 3), has been reported only once before (Antonov et al. 2000) and is difficult to evaluate, particularly given the current controversy surrounding the confidence one can have in the PP in Bayesian phylogenetics (Suzuki et al. 2002; Douady et al. 2003; Felsenstein 2004; Simmons et al. 2004).

The placement of Chloranthaceae among other basal angiosperms has long been a subject of debate (Qiu et al. 1993). Our nine-gene and five-protein-gene analyses did not yield bootstrap support to place this family with confidence (figs. 1, 2). Clearly, more work is needed to determine the phylogenetic affinity of this family.

Relationships among magnoliids, monocots, and eudicots, the three lineages encompassing nearly 3%, 22%, and 75% of all angiosperm species diversity, respectively (Drinnan et al. 1994), continue to elude resolution despite several large-scale sequence analyses (Soltis et al. 2000; Savolainen et al. 2000; Hilu et al. 2003). Monocots were placed in a clade with magnoliids and Chloranthaceae with 56% jackknife support in Soltis et al. (2000), and this topology was also recovered by Hilu et al. (2003) using a different data set in a Bayesian analysis (but not in their parsimony analysis). Eudicots-Ceratophyllum were sister to this large clade. Our Bayesian analysis of the five-protein-gene matrix obtained a similar topology, with Ceratophyllum placed as a sister to Chloranthaceae instead of eudicots (fig. 3). Alternatively, the eudicots-Ceratophyllum clade is sister to the magnoliids in our five-protein-gene parsimony analysis, but without bootstrap support >50% (fig. 2). A similar topology was obtained in our earlier studies using a slightly different data set (Qiu et al. 1999, 2000) with the exception that Ceratophyllum was not placed with eudicots but rather with monocots. The third possible arrangement for these three large angiosperm lineages, with monocots and eudicots as sister to each other, has been seen in three analyses of plastid and nuclear genes (Mathews and Donoghue 1999; Graham and Olmstead 2000b; Graham et al., forthcoming). Thus, all three possible arrangements for monocots, magnoliids, and eudicots have been observed. It is clear that more data, in terms of both character and taxon sampling (particularly of monocots and eudicots), are needed before a firm conclusion can be reached on relationships among these three large angiosperm lineages.

**Conclusions**

Our analyses, as well as several earlier studies of the angiosperm phylogeny, revealed a steady increase in resolution and internal support for relationships as genes were added to initial single-gene matrices to form multigene data sets. For example, Soltis et al. (1998) revealed a steady increase in support for angiosperm relationships (including basal angiosperm 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 compared to the five-gene analysis of Qiu et al. (1999), based on atpB, 18S, rbcL, atp1, and matR, involves the signal provided by matK. In fact, the rapidly evolving matK alone provides resolution and support comparable to that achieved with three more slowly evolving genes, rbcL, 18S, and atpB (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 example, the addition of 18S did increase the support for the placement of Amborella as sister to all other flowering plants, but conversely, the support for the magnoliid clade was somewhat lower than that achieved with the five protein-coding genes. The addition of 26S slightly increased support for the placement of Amborella, but support for the monophyly of the magnoliid clade and 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
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-genome 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; Dombrovksa and Qiu 2004; Qiu and Palmer 2004; 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 homological 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 heterogenous (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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