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BASAL ANGIOSPERM PHYLOGENY INFERRED FROM DUPLICATE PHYTOCHROMES A AND C

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We have extended our studies of angiosperm phylogeny based on a pair of duplicated phytochrome (*PHY*) genes, *PHYA* and *PHYC*. Phylogenetic analyses of sequences from 52 species yield unrooted gene networks in which all of the elements resolved in our previous analysis of 26 species appear. *Amborella* still emerges as the sister group of all other angiosperms. However, we cannot reject alternative rootings in which water lilies, either alone or in combination with *Amborella*, are basal. *Austrobaileya* + *Illicium* diverges next from the remaining angiosperms. Eudicots and monocots form rather well-supported clades, as do Magnoliales, Laurales, Piperales, and winteroids, but relationships among these major lineages remain uncertain, as do the positions of Chloranthaceae and *Ceratophyllum*. Magnoliales may be directly linked with Laurales and Piperales with winteroids, but support for these relationships is not strong. Within eudicots, a basal split between ranunculids (Ranunculales, Papaverales) and the rest of the eudicots is supported, though the position of *Nelumbo* is equivocal. These same relationships are obtained in combined analyses of *PHYA* and *PHYC* (species as terminals) when *Ceratophyllum* is excluded. However, when *Ceratophyllum* is included, *Austrobaileya* + *Illicium* and then Chloranthaceae diverge from the remaining angiosperms before *Ceratophyllum* + water lilies. Rooted species trees inferred from duplicate gene networks by minimizing gene duplications and losses are highly congruent with the gene subtrees and with the results of recent analyses of other genes, even when *Ceratophyllum* is included. More attention must be paid to the methods for obtaining rooted species trees from data sets that include duplicate genes, especially if we are to fully implement the search for species trees that simultaneously minimize the multiple possible causes of conflict among gene trees.

Keywords: angiosperm phylogeny, duplicate genes, duplicate gene rooting, phytochrome genes, uninode coding.

Introduction

In two previous analyses we explored the use of a gene duplication in the phytochrome gene family, leading to *PHYA* and *PHYC*, in order to root the phylogeny of angiosperms (Donoghue and Mathews 1998; Mathews and Donoghue 1999). Our first analysis of data from just 12 species resolved highly congruent gene subtrees, demonstrating the potential of the approach, but it lacked many potentially basal lineages. Sampling was improved in our subsequent analysis, and we obtained a more convincingly rooted species tree. However, many major clades within angiosperms were represented by just one (e.g., Chloranthaceae) or a few (e.g., monocots and eudicots) species. Here we report results of similar analyses of *PHYA* and *PHYC* sequences from nearly twice as many species.

Background

Phylogenetic analyses of phytochrome genes in green plants indicate that the phytochrome gene pair *PHYA* and *PHYC* diverged prior to the origin of angiosperms (Mathews and Sharrock 1997; Donoghue and Mathews 1998). *PHYA* and *PHYC* are found in most angiosperms examined (but see Howe et al. 1998; Lavin et al. 1998) and form well-supported clades (bootstrap values >90%), indicating that they are evolving independently (Mathews and Sharrock 1997). A single-gene lineage related to this pair is known from other seed plants (Mathews and Sharrock 1997; Mathews and Donoghue 1999); this gene lineage has diversified in conifers (Clapham et al. 1999; S. Mathews, unpublished data) and perhaps in other seed plants.

In most angiosperms, phytochrome genes occur as a small family comprising three to five members. The completely characterized phytochrome family of *Arabidopsis* has five members, *PHYA* through *PHYE* (Sharrock and Quail 1989; Clack et al. 1994). Typical phytochrome coding sequences range from ca. 3.3 to ca. 4.1 kb and are interrupted by three introns, a structure shared by most land plant *PHY* (Cowl et al. 1994; Quail 1994; Wada et al. 1997). Amino acid sequences inferred from the five *Arabidopsis PHY* are 46%–80% identical; *PHYA* and *PHYC* are 52% identical (Sharrock and Quail 1989; Clack et al. 1994). The five encoded photoreceptors have both distinct and overlapping roles in photomorphogenesis. Phytochrome A (phyA) controls hypocotyl elongation, cotyledon expansion, and seed germination in response to prolonged far-red irradiation; phyA also mediates responses to single pulses of far-red light or very low doses of red light (Whitelam and Devlin 1997). Overexpression of phyC in transgenic *Arabi-*

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dopsis indicates that its photosensory specificity is distinct from that of phyA, being more closely similar to that of phyB, and that phyC has a capacity distinct from that of both phyA and phyB to enhance leaf expansion in white light (Qin et al. 1997).

Our original analyses of sequences from 12 angiosperms resolved an unrooted network of two gene subtrees that shared eight identical components (Donoghue and Mathews 1998). Many pertinent taxa (e.g., Piperales, Nymphaeaceae, *Amborella*) were not sampled, and the branch connecting the gene subtrees rooted each subtree near the highly divergent taxon *Sorghum*. Clades of eudicots and of Magnoliales + Laurales were resolved in both subtrees. Duplicate gene networks were more resolved than single-gene trees from analyses with outgroup sequences. These data were subsequently analyzed by Simmons et al. (2000) in a variety of ways: uncombined (sequences as terminals), combined (species as terminals) via "uninode" coding (see below), and combined with *rbc*L and 18S data. Simmons et al. (2000) obtained results very similar to those found by Donoghue and Mathews (1998). For example, the *PHYA* and *PHYC* subtrees resolved in their analysis with outgroups are identical to those inferred from our analysis without outgroups. Their uninode matrix yielded a species tree identical to the *PHYC* subtree, and the rooting at *Sorghum* was retained when the phytochrome data were combined with *rbc*L and 18S nuclear ribosomal data (Simmons et al. 2000).

Subsequently, we obtained and analyzed *PHYA* and *PHYC* sequences from 26 angiosperms (Mathews and Donoghue 1999) that represented most of the taxa previously suggested to be early diverging lineages. As in the analysis of 12 species, analyses of *PHYA* and *PHYC* singly with outgroups did not resolve the root (S. Mathews, unpublished data). But in the strict consensus of duplicate gene networks, both gene subtrees showed a basal split separating *Amborella* from all other angiosperms. The position of *Amborella* was strongly supported in both *PHYA* and *PHYC* subtrees (92% and 83% bootstrap support, respectively, for the remaining angiosperms). *Austrobaileya* branched next from the remaining angiosperms in the *PHYA* subtree, with moderate support (66% for the remaining angiosperms), whereas *Nymphaea* + *Cabomba*, or a clade including all three taxa, branched next in the *PHYC* subtree. However, the remaining angiosperms were similarly resolved in the subtrees of the network that maximized identical components in the *PHYA* and *PHYC* subtrees (Mathews and Donoghue 1999, fig. 1). In both subtrees, winteroids were sister to Piperales in a magnoliid clade, and eudicots were united with monocots + *Chloranthus*. Magnoliales and Laurales either formed a clade (*PHYA* subtree) or were paraphyletic with respect to winteroids + Piperales (*PHYC* subtree). When data from *PHYA* and *PHYC* were combined (species as terminals), greater resolution was obtained. In the resulting tree, which was rooted along the branch to *Amborella*, Nymphaeales diverged first from the remaining angiosperms, followed by *Austrobaileya* (supported by bootstrap values of 80% and 86%, respectively, for the remaining angiosperms). The combined data also better supported (bootstrap values >60%) some of the clades that were resolved in the gene subtrees, including the magnoliid clade, and clades uniting winteroids with Piperales and Laurales with Magnoliales. Several other recent analyses (Parkinson et al. 1999; Qiu et al. 1999; Soltis et al. 1999; Doyle and Endress 2000; Graham et al. 2000; Soltis et al. 2000) have produced similar results.

Material and Methods

Our current analyses include both *PHYA* and *PHYC* sequences from 48 taxa, plus *PHYA* only from *Tacca* and *PHYC* only from *Illicium*, *Lemna*, and *Pleea* (table 1). From Cabombaceae, we sampled *PHYA* from *Brasenia* but *PHYC* from *Cabomba*. Phytochrome loci were sampled using primers and protocols described previously (Mathews and Donoghue 1999); stepdown gene amplification protocols (Hecker and Roux 1996) with starting annealing temperatures of 60°C to 65°C were most effective. Fragments of exon 1 (1.2–1.4 kb) or of exons 1 and 2 and the intervening intron (1.6–1.8 kb) were amplified, cloned, and sequenced.

Each cloned sequence was aligned with all other available phytochrome sequences from land plants. The alignment was edited, and final data matrices of 3255 nucleotide sites from all four exons and of 1303 nucleotide sites from exon 1 were constructed using Se-Al (Rambaut 1996). Three alignments were analyzed. In the first two alignments, sequences are terminals, whereas in the third, species are terminals. The first (3255 nucleotide sites) was a comparison of all duplicated and unduplicated sequences to infer a gene phylogeny. From this we determined whether newly obtained data altered the interpretation (of previous studies) that *PHYA* and *PHYC* are monophyletic gene lineages and that they diverged along the branch to angiosperms (e.g., Mathews et al. 1995; Mathews and Sharrock 1997). The second alignment (1303 nucleotide sites) included only *PHYA* and *PHYC* sequences obtained from the species listed in table 1, which were analyzed to obtain an unrooted gene network of two gene subtrees. We used GeneTree (Page 1998) to identify optimal species trees compatible with most parsimonious gene networks inferred in analysis of the second alignment. Ten random starting trees were rearranged by alternating subtree pruning and regrafting and nearest neighbor interchange to find species trees into which the gene networks fit with the fewest number of duplications and losses (see "Discussion"). Equally good solutions were retained, and the steepest descent option was in effect.

If *PHYA* and *PHYC* are evolving independently, they can be combined to obtain a species phylogeny. In the third alignment (2606 nucleotide sites), species are terminals, and the data from each gene are concatenated. The *PHYA* from *Brasenia* and the *PHYC Cabomba* were combined in a single terminal, Cabombaceae, in this alignment. The root of the species phylogeny was positioned according to evidence from the gene networks inferred in analysis of the second alignment.

Parsimony analyses using PAUP* version 4.0b3a or 4.0b4a (Swofford 2000) comprised heuristic searches of 100 replicates of random taxon addition with tree bisection and reconnection (TBR) branch swapping. We used two character-weighting schemes, one in which characters were equally weighted and one in which third codon positions were assigned a weight of 0.75. Bootstrap values (Felsenstein 1985) were estimated in 100 replicates of heuristic searches with 10 replicates of random taxon addition and TBR branch swapping. We conducted analyses with and without the sequences from *Ceratophyllum*

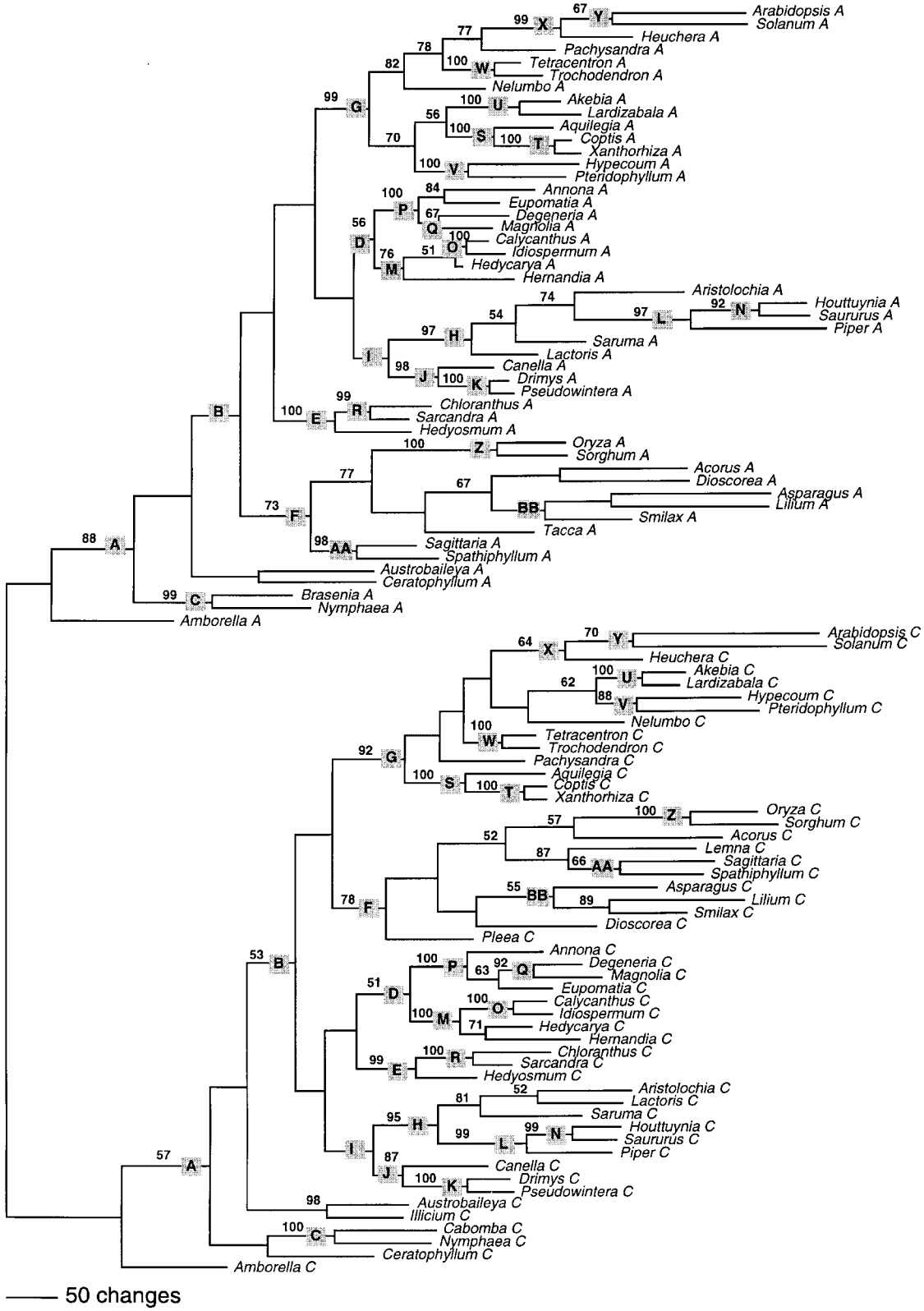


Fig. 1 One of 10 most parsimonious networks of *PHYA* and *PHYC* from 52 angiosperms (1303 nucleotide sites, 838 parsimony informative) that maximizes identical components in the gene subtrees; weighted parsimony analysis (100 random taxon addition replicates with TBR swapping in PAUP* 4.0 [Swofford 2000]) yielded networks of 10,875.25 steps (retention index [RI] = 0.54; consistency index [CI] = 0.15, excluding autapomorphies). Bootstrap percentages (from 100 replicates with the same search parameters, but using 10 random addition replicates) are shown above branches. Identical components in the *PHYA* and *PHYC* subtrees are labeled *A* through *BB*.

Table 1

Species Sampled for Phytochrome Data, Voucher Information, and GenBank Accession Numbers

Clade	Species	Voucher	GenBank accession numbers	
			PHYA	*PHYC*
Amborellaceae	*Amborella trichopoda* Baill.	*M. P. Simmons 1846* LH	AF190062	AF190063
Austrobaileyaceae	*Austrobaileya scandens* C. T. White	*SYM 388* A	AF190068	AF190069
Canellaceae	*Canella winterana* Gaertner	A-6087-FTG	AF190074	AF190075
Winteraceae	*Drimys winteri* J. R. & G. Forst.	*SYM 397* A	AF190080	AF190081
	Pseudowintera axillaris Dandy	*SYM 412* A	AF276737	AF276738
Chloranthaceae	*Chloranthus spicatus* Mak.	*A. Bernhard 176-96*	AF190076	AF190077
	Hedyosmum Sw. sp.	nv; BGSC	AF276722	AF276723
	Sarcandra glabra (Thunb.) Nakai	*SYM 419* A	AF276741	AF276742
Illiciaceae	*Illicium oligandrum* Merr. and Chun	*SYM 450* A	nd	AF276729
Nymphaeaceae	*Brasenia schreberi* J. F. Gmelin	nv; HUBL	AF190070	nd
	Cabomba Aublet sp.	nv; HUBL	nd	AF190071
	Nymphaea odorata Aiton	*SYM 392* A	AF190098	AF190099
Ceratophyllales	*Ceratophyllum demersum* L.	*SYM s.n.* MONT	AF276716	AF276717
Laurales	*Calycanthus floridus* L.	*SYM 404* A	AF190072	AF190073
	Hedycarya angustifolia A. Cunn.	NSW 410584 A	AF190084	AF190085
	Hernandia L. sp.	*Alverson & Rubio 2238* A	AF190086	AF190087
	Idiospermum australiense S. T. Blake	NSW 410587 A	AF190089	AF190090
Magnoliales	*Annona* L. sp.	nv; HUBL	AF190064	AF190065
	Degeneria vitiensis L. W. Bailey & A. C. Smith	*John Miller 1189-63*	AF190078	AF190079
	Eupomatia laurina Hook.	NSW 410586 A	AF190082	AF190083
	Magnolia × *soulangiana* Hort. [ex Thieb.]	*SYM 460* A	AF190094	AF190095
Piperales	*Aristolochia grandiflora* Sw.	*SYM 399* A	AF276712	AF276713
	Houttuynia cordata Thunb.	*A. Bernhard 174-96*	AF276726	AF190088
	Lactoris fernandeziana Phil.	*MWC 1014* K	AF190091	AF190092
	Piper nigrum L.	*SYM 401* A	AF190100	AF190101
	Saururus cernuus L.	*A. Bernhard 176-96*	AF190106	AF190107
	Saruma henryi Oliver	*LK 1034* LH	AF190104	AF190105
Monocots:				
Acorales	*Acorus gramineus* [Soland in] Ait. Hort. Kew.	*S. Mathews 413* A	AF190060	AF190061
Alismatales	*Lemna gibba* L.	nv via Elaine Tobin	nd	AF190093
	Spathiphyllum "Clevelandii"	*S. Mathews 400* A	AF276745	AF276746
	Sagittaria L. sp.	*S. Mathews 383* A	AF190102	AF190103
Asparagales	*Asparagus falcata*	*W. J. Hahn 6881* WIS	AF276714	AF276715
Dioscoreales	*Dioscorea elephantipes* Engl.	*S. Mathews 425* A	AF276720	AF276721
	Tacca chantrieri Andre	nv; BGSC	AF276747	nd
Liliales	*Lilium superbum* L.	*W. J. Hahn s.n.* WIS	AF276732	AF276733
	Pleea tenuifolia Michaux	*M. W. Chase 152* K	nd	AF276736
	Smilax rotundifolia L.	*S. Mathews 452* A	AF276743	AF276744
Poales	*Oryza sativa* L.	Published data	X14172	AB018442
	Sorghum bicolor Moench	Published data	U56729	U56729
Eudicots:				
Buxaceae	*Pachysandra* Michaux sp.	*S. Mathews 431* A	AF276734	AF276735
Trochodendraceae	*Tetracentron sinense* Oliver	*S. Mathews 423* A	AF276748	AF276749
	Trochodendron aralioides Siebold & Zucc.	*S. Mathews 424* A	AF190108	AF190109
Proteales	*Nelumbo nucifera* Gaertner	nv; HUBG	AF190096	AF190097

Table 1

(*Continued*)

Clade	Species	Voucher	GenBank accession numbers *PHYA*	GenBank accession numbers *PHYC*
Ranunculales	*Akebia quinata* (Houtt.) Decne.	*S. Mathews 446* A	AF276710	AF276711
	Aquilegia L. sp.	nv	AF190066	AF190067
	Coptis trifolia (L.) Salisb.	*S. Mathews 459* A	AF276718	AF276719
	Hypecoum imberbe Sm.	*M. W. Chase 528* K	AF276727	AF276728
	Lardizabala biternata Ruiz & Pav.	*S. Mathews 405* A	AF276730	AF276731
	Pteridophyllum racemosa Siebold and Zucc.	*M. W. Chase 531* K	AF276739	AF276740
	Xanthorhiza simplicissima Marshall	*S. Mathews 458* A	AF276750	AF276751
Saxifragales	*Heuchera* L. sp.	*S. Mathews 457* A	AF276724	AF276725
Brassicales	*Arabidopsis thaliana* (L.) Heynh.	Published data	L21154	X17343
Solanales	*Solanum lycopersicon* L.	Published data	AJ001915	AF178568

Note. Clade arranged according to APG (1998). nd = not determined; nv = no voucher; BGSC = Botanic Garden of Smith College, courtesy of R. Nicholson; HUBL = Harvard University Biological Laboratories, courtesy of A. MacDonald or P. F. Stevens. Alignments of these sequences are available in TreeBase (http://phylogeny.harvard.edu/treebase) under accession number SN543.

because it is the only taxon in our data set for which there is evidence of multiple *PHYA* or *PHYC* (Mathews et al. 1995). In our current study, we have amplified just one of its two *PHYA*, and we could not fully explore its history in the absence of the other. If duplication in the *PHYA* lineage occurred before the origin of *Ceratophyllum*, the possibility exists of comparing paralogous rather than orthologous *PHYA* sequences.

Differences in the *PHYA* and *PHYC* subtrees that do not result merely from inadequate signal might result from (1) sampling error (in this case, either from inadequate taxonomic sampling or undetected paralogy), (2) different processes (such as functional constraints) acting on the characters, and (3) different branching histories of the sampled sequences (de Quieroz et al. 1995). We used partition homogeneity tests (Farris et al. 1994, 1995) to determine whether processes affecting *PHYA* and *PHYC* might differ. Partition homogeneity tests were implemented with PAUP* using the same heuristic search settings as were used in the bootstrap analyses. Taxa from which a single gene was sampled were excluded from these analyses so that no species had half of its characters coded as missing. We used paired tests of specific tree topologies to assess whether conflicting branching orders were well supported by the data. Using the nonparametric Templeton test (Larson 1994), implemented with PAUP*, we compared (1) the *PHYA* and *PHYC* subtrees resolved in the gene network that was inferred in analyses of the second alignment, (2) the *PHYA* subtree with sets of *PHYA* trees in which branching order was constrained to match individual nodes of the *PHYC* subtree, and (3) the *PHYC* subtree with sets of *PHYC* trees in which branching order was constrained to match individual nodes of the *PHYA* subtree. We also used the Templeton test to compare gene networks inferred in unconstrained analysis of the second alignment with those from analyses in which alternative rootings of the subtrees were enforced.

Results

Analyses in Which Sequences Are Terminals

Analysis of the 49 *PHYA* and the 51 *PHYC* sequences resulted in eight or two most parsimonious duplicate gene networks in unweighted or weighted parsimony analyses, respectively. The consensus trees from searches with and without weights are nearly identical, differing only with respect to the position of the Piperales in the *PHYC* subtree. In the consensus from the unweighted search, Piperales are united in a polytomy with eudicots and monocots. In the consensus of the weighted search, they are united with winteroids. Of the 10 shortest networks from both searches, one of the two inferred in the weighted search maximizes identical components in the two gene subtrees and is depicted in figure 1. All of the elements resolved in our analyses of 26 angiosperms appear in this tree. *Amborella* diverges first from the remaining angiosperms, followed by Nymphaeales + *Ceratophyllum* and then by *Austrobaileya* + *Illicium* in the *PHYC* subtree or by Nymphaeales and then by *Austrobaileya* + *Ceratophyllum* in the *PHYA* subtree. As before, within the remaining angiosperms, a magnoliid clade is resolved in which Magnoliales (P) are sister to Laurales (M) and winteroids (J) are sister to Piperales (H), as are clades of monocots (F) and eudicots (G). However, a few elements are differently placed. For example, in the *PHYA* subtree, monocots (F) are sister to the remaining angiosperms. This conflicts with their position in the *PHYC* subtree and with our earlier results, which placed them as sister to the eudicots. Chloranthaceae (E) are also differently resolved, as sister to Magnoliales + Laurales (D) in the *PHYC* subtree or as sister to the magnoliids + eudicots in the *PHYA* subtree. In our analysis of 26 angiosperms, Chloranthaceae was represented by *Chloranthus* only and was sister to the monocots (Mathews and Donoghue 1999).

In general, nodes that were well supported (bootstrap values above 70%) in the analysis of 26 angiosperms are also supported in analyses of the larger phytochrome data set. For example, Nymphaeales (C), Magnoliales (P), winteroids (J), and Piperales (H) are supported by bootstrap values of 87%–100% and monocots (F) by values of ≥73% (fig. 1). In some cases, additional sampling resulted in better resolution, as was the case with eudicots (G), which are now supported by bootstrap values of 92% and 99%, respectively, in the *PHYC* and *PHYA* subtrees (compared with 81% and 96%, respectively, in the analysis of 26 species). Laurales (M) are better supported by the *PHYA* data in the larger analysis, with a bootstrap value of 76% (compared with 57% in the analysis of 26 species), whereas support by the *PHYC* data remains high (100% in both analyses). The larger analysis supports the monophyly of the Chloranthaceae (E), with bootstrap values of 99% and 100%, respectively, in the *PHYC* and *PHYA* subtrees. The placement of the newly sampled *Illicium PHYC* with *Austrobaileya PHYC* is supported by a bootstrap value of 98%. In some cases, however, additional sampling resulted in decreased resolution. Specifically, the position of *Amborella* remains well supported in the *PHYA* subtree (bootstrap value of 88%, compared with 92% in the analysis of 26 species) but is less well supported in the *PHYC* subtree (bootstrap value of 57%, compared with 83% in the analysis of 26 species).

Exclusion of the sequences of *Ceratophyllum* has a marked effect on resolution of the early divergences in each subtree (fig. 2), but it has a limited impact elsewhere in the subtrees. In both subtrees, the position of *Amborella* is relatively well supported (82% and 74%, respectively, for the remaining angiosperms in the *PHYA* and *PHYC* subtrees). In the *PHYC* subtree, Nymphaeales diverge next from the remaining angiosperms, followed by *Austrobaileya* + *Illicium* (76% and 72%, respectively, for the remaining angiosperms). In the *PHYA* subtree, *Austrobaileya* diverges next from the remaining angiosperms, followed by Nymphaeales (65% and 51%, respectively, for the remaining angiosperms). When *Ceratophyllum* is excluded, the *PHYC* subtree differs in placing Chloranthaceae (E) as sister to the remaining angiosperms. The *PHYA* subtree differs when *Ceratophyllum* is excluded because it places Chloranthaceae (E) as sister to the eudicots (G) and because Piperales (H), winteroids (J), and magnoliids (P) are paraphyletic with respect to eudicots + Chloranthaceae (fig. 2).

In both analyses, relationships within the eudicot clade are well supported in the *PHYA* subtree (nine of 11 clades with ≥70% bootstrap values). The subtree diverges into two principal clades, one of ranunculids (*sensu* Hoot et al. 1999), in which Papaverales (V) are united with Ranunculaceae (S) + Lardizabalaceae (U), and one in which *Nelumbo*, Trochodendrales (W), and *Pachysandra* are paraphyletic with respect to the core eudicots (X) (figs. 1, 2). In the *PHYC* subtrees, the ranunculids do not form a clade, and Papaverales (V) are united with Lardizabalaceae (U). The latter relationship receives moderate (62%) or little (<50%) support in analyses that include or exclude (respectively) *Ceratophyllum*.

To assess the degree of support in the phytochrome data for alternative rootings, we compared networks rooted near *Amborella* with those resulting from nine searches with different constraints enforced. Individual constraint trees enforced rootings of both subtrees near Nymphaeales + *Amborella*, Nymphaeales alone, *Austrobaileya*, Magnoliales, Laurales, Piperales, Chloranthaceae, winteroids, or monocots. In paired tests, all of the networks resulting from constrained searches are significantly worse ($P < 0.0001$–0.0089 using the Templeton test) than the shortest networks found in unconstrained searches, except for the networks constrained so that each subtree was rooted near Nymphaeales or near Nymphaeales + *Amborella*. Thus, all rootings other than those near *Amborella* or Nymphaeales, or near the two together, are rejected by the phytochrome data.

None of the conflicting nodes resolved by the *PHYA* and *PHYC* subtrees is supported above 50% in bootstrap analyses (figs. 1, 2). However, paired tests revealed that the *PHYA* data reject the *PHYC* tree and that the *PHYC* data reject the *PHYA* tree, regardless of whether *Ceratophyllum* is included in the analyses ($P < 0.0001$ for both pairwise comparisons using the Templeton test). Furthermore, partition homogeneity tests reject the hypothesis that the single-gene data sets are homogeneous ($P = 0.02$). The latter result may reflect the fact that *PHYA* apparently is evolving significantly faster than *PHYC* in most of the species sampled (S. Mathews, unpublished data).

We used constraint analyses to determine whether this conflict could be attributed to specific nodes of the subtrees. Specifically, we constrained three searches of the *PHYA* data such that in each, the resulting tree matched the *PHYC* subtree with respect to the placement of one of the following: (1) monocots, (2) Chloranthaceae, or (3) *Ceratophyllum*. Likewise, we constrained three searches of the *PHYC* data such that in each, the resulting tree matched the *PHYA* subtree with respect to placement of one of the same three taxa. The resulting sets of trees were compared with the *PHYA* and *PHYC* subtrees, respectively, from unconstrained analyses. None of the trees resulting from constraint analyses is rejected by the *PHYA* or *PHYC* data ($P > 0.1$ for all pairwise comparisons using the Templeton test).

Although we cannot attribute the conflict that we observed to any of the single nodes that we tested, the evidence that we obtained supporting significant conflict in more general tests might serve as an argument against combining data from the genes (e.g., Bull et al. 1993). Nonetheless, we conducted a combined analysis in order to compare the results with those obtained in gene tree parsimony analyses.

Analyses in Which Species Are Terminals

When *Ceratophyllum* is included, both weighted and unweighted parsimony analyses of the combined data resolved the same set of three most parsimonious trees, the consensus of which is depicted in figure 3. Basal relationships are poorly supported by bootstrap values (<50%) and differ in two ways from those resolved in the gene subtrees that included *Ceratophyllum*. *Austrobaileya* + *Illicium*, rather than Nymphaeales, diverge from the remaining angiosperms after *Amborella*, and Chloranthaceae diverge next. Nymphaeales + *Ceratophyllum* are sister to the remaining angiosperms. This branching order is surprising because Nymphaeales branched before *Austrobaileya* in all *PHYC* subtrees and in some *PHYA* subtrees. Moreover, none of the subtrees resolved Chloranthaceae as diverging before Nymphaeales (e.g., figs. 1, 2). The remaining angiosperms are resolved as they were in the *PHYC*

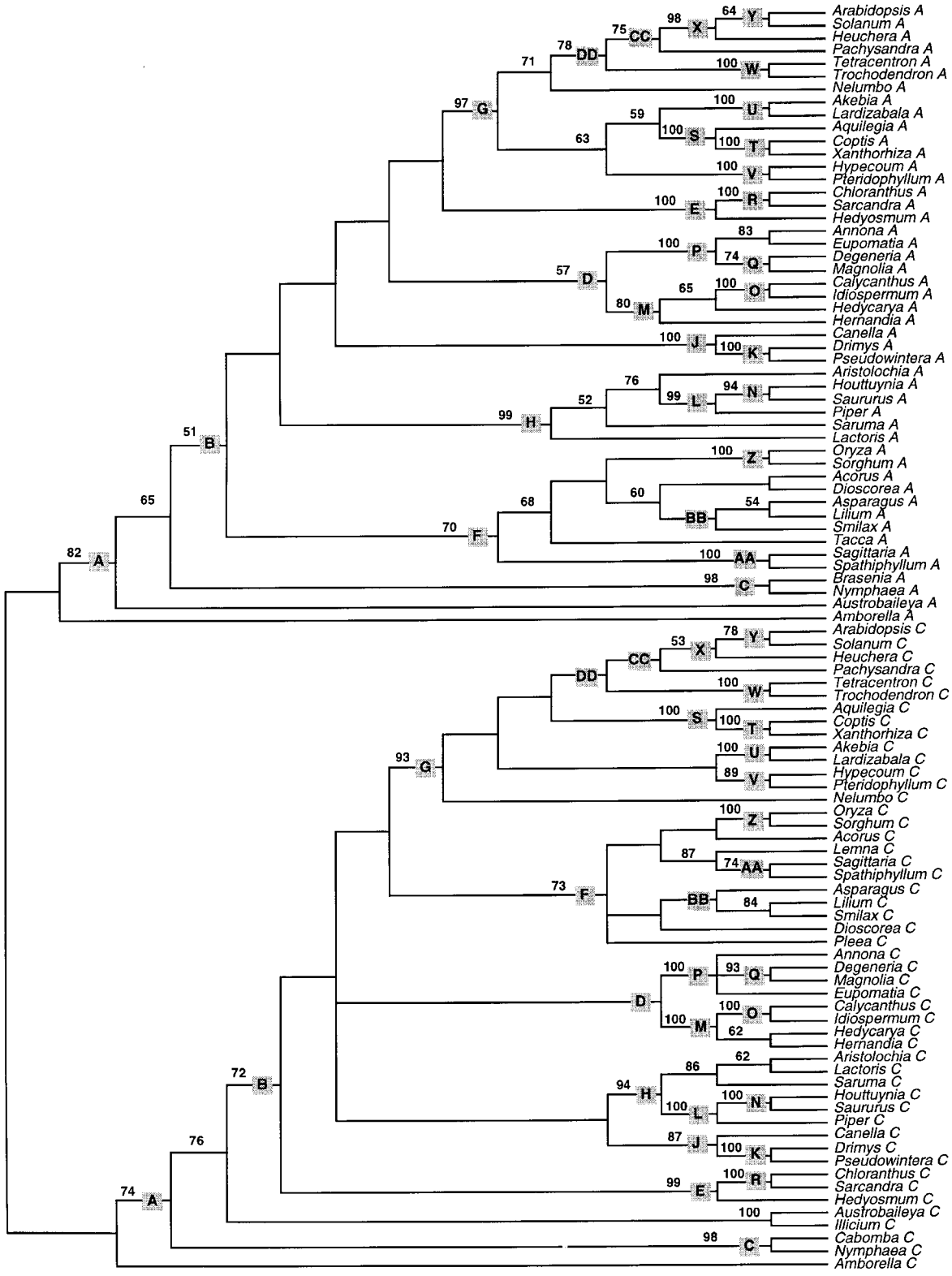


Fig. 2 Strict consensus of two most parsimonious networks of *PHYA* and *PHYC* from 51 angiosperms (*Ceratophyllum* excluded; 1303 nucleotide sites, 834 parsimony informative); weighted parsimony analysis (100 random taxon addition replicates with TBR swapping in PAUP* 4.0 [Swofford 2000]) yielded most parsimonious networks of 10,575.00 steps (retention index [RI] = 0.54; consistency index [CI] = 0.16, excluding autapomorphies). Bootstrap percentages (from 100 replicates with the same search parameters, but using 10 random addition replicates) are shown above branches. Identical components in the *PHYA* and *PHYC* subtrees are labeled *A* through *DD*.

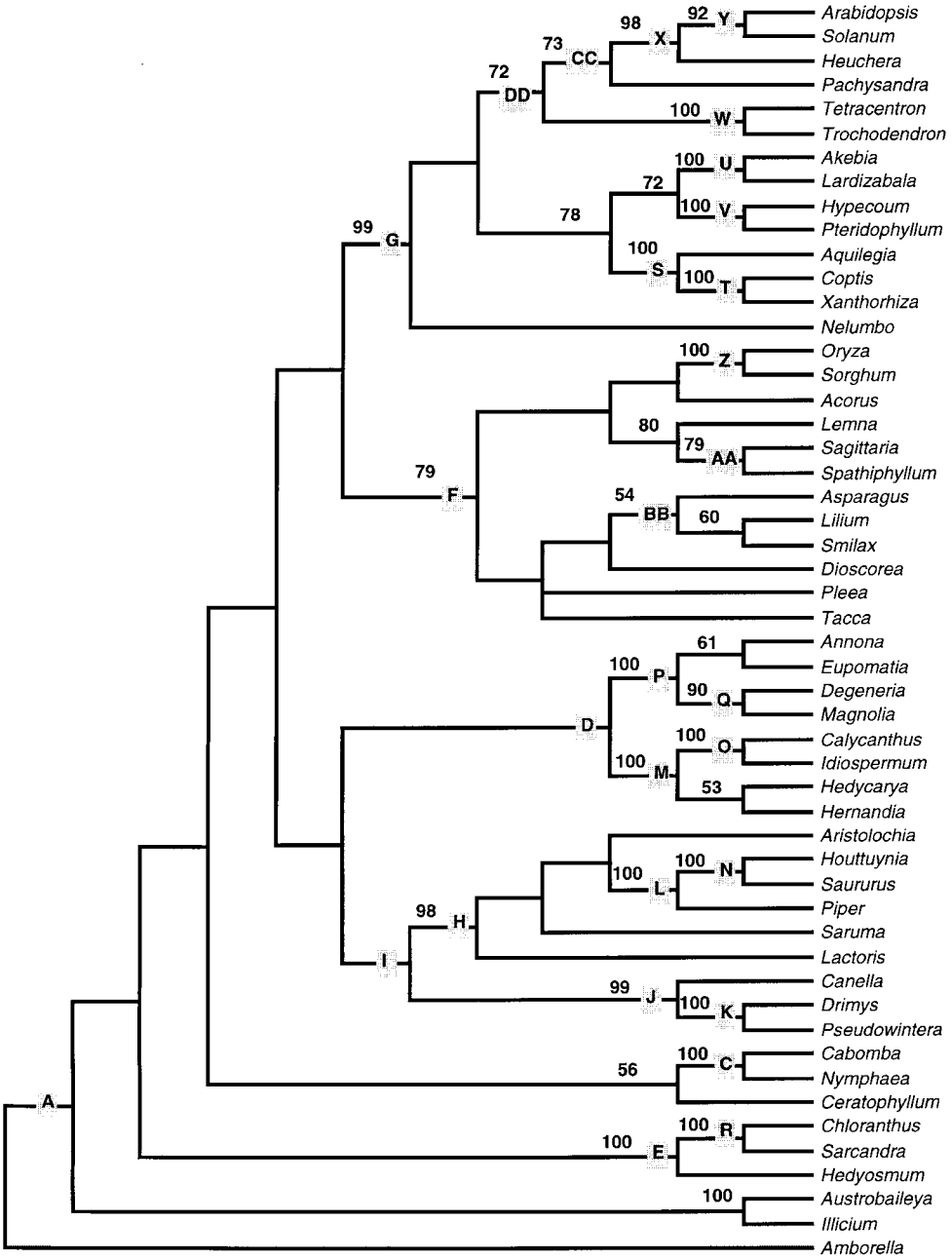


Fig. 3 Strict consensus of three most parsimonious trees from combined analysis of *PHYA* and *PHYC*, with species as terminals (2606 nucleotide sites; 1408 parsimony informative), rooted near *Amborella*, based on the duplicate gene analysis (figs. 1, 2); weighted parsimony analysis (100 random taxon addition replicates with TBR swapping in PAUP* 4.0 [Swofford 2000]) yielded trees of 11,821.75 steps (retention index [RI] = 0.41; consistency index [CI] = 0.23, excluding autapomorphies). Bootstrap percentages (from 500 replicates with the same search parameters but using 10 random addition replicates) are shown above branches. Components identical to those found in both *PHYA* and *PHYC* subtrees are labeled *A* through *DD*.

subtree of figure 1 and in Mathews and Donoghue (1999). Overall, this result contrasts with results from our analyses of 26 angiosperms, in which combining the data led to better support for the earliest divergences as well as for some of the major clades (Mathews and Donoghue 1999). Constraint analyses reveal that the tree from the combined data is rejected by the *PHYA* data ($P < 0.0001$ using the Templeton test) but not by the *PHYC* data ($P > 0.3247$).

As in our analyses of the sequences as terminals, excluding *Ceratophyllum* from analyses of the combined data resulted in better resolution at the base of the tree (fig. 4). After *Amborella*, Nymphaeales diverge first from the remaining angio-

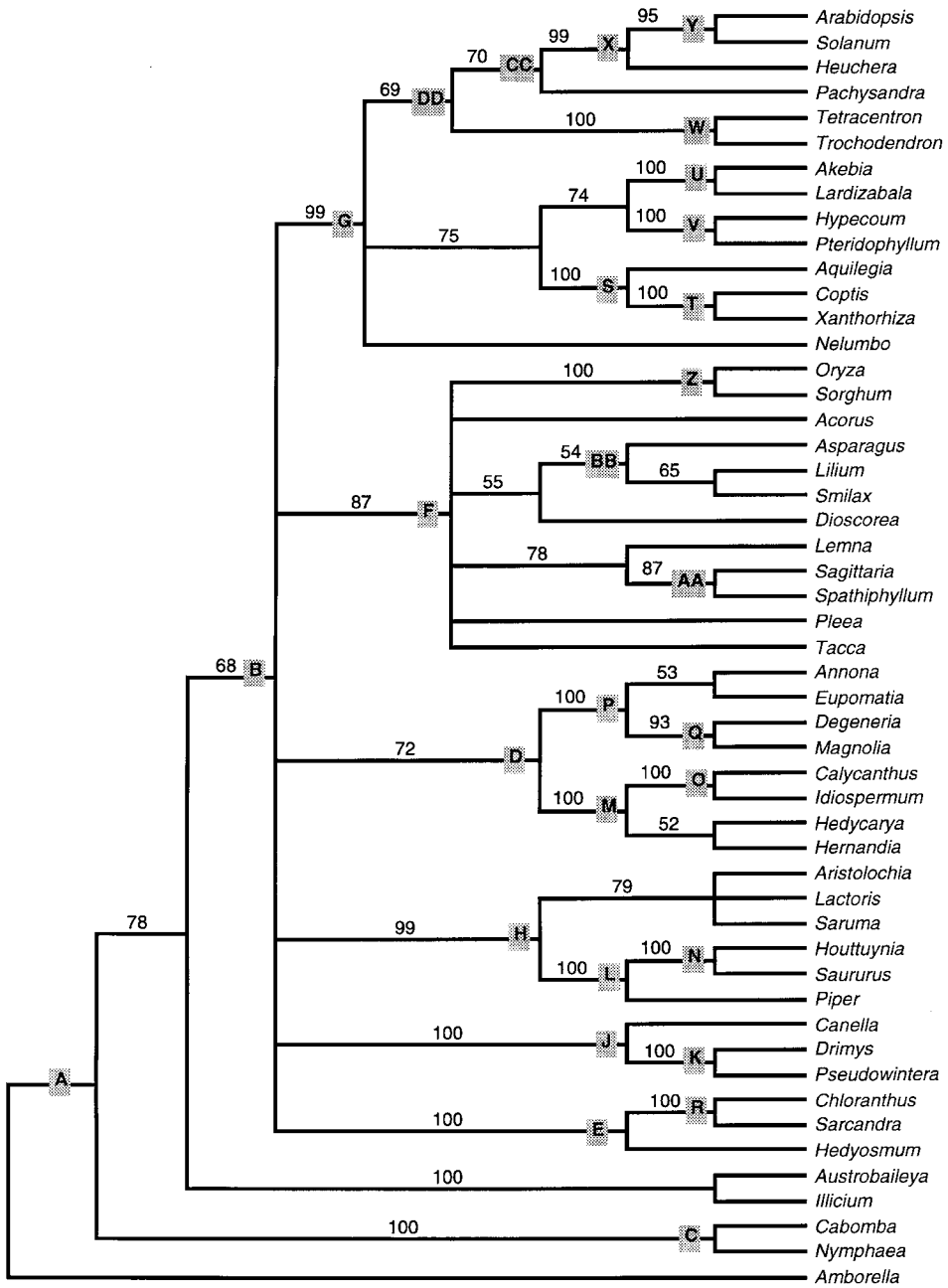


Fig. 4 Bootstrap consensus (50% majority rule) from combined analysis of *PHYA* and *PHYC*, with sequences as terminals (2606 nucleotide sites; 1399 parsimony informative) when *Ceratophyllum* is excluded, rooted near *Amborella*, based on the duplicate gene analysis (figs. 1, 2); weighted parsimony analysis (100 random taxon addition replicates with TBR swapping in PAUP* 4.0 [Swofford 2000]) yielded trees of 11,495.50 steps (retention index [RI] = 0.42; consistency index [CI] = 0.23, excluding autapomorphies). Bootstrap percentages (from 100 replicates with the same search parameters but using 10 random addition replicates) are shown above branches. Components identical to those found in both *PHYA* and *PHYC* subtrees are labeled *A* through *DD*.

sperms, then from *Austrobaileya* + *Illicium*. And, as in our analyses of 26 angiosperms, the *PHYA* and *PHYC* subtrees without *Ceratophyllum* conflict with respect to the position of *Austrobaileya* (fig. 2), but the combined data (fig. 4) support the separation of Nymphaeales first (bootstrap value of 78% for the remaining angiosperms), followed by *Austrobaileya* + *Illicium* (bootstrap value of 68% for the remaining angiosperms). In general, clades within the remaining angiosperms are supported at similar levels in analyses with and without *Ceratophyllum*. For, example, eudicots (G), Piperales (H), winteroids (J), Magnoliales (P), Laurales (M), and Chloranthaceae (E) are supported by bootstrap values ≥95%. However, a few clades are better supported when *Ceratophyllum* is excluded, including monocots (F), Magnoliales + Laurales

(D), and Aristolochiales (bootstrap values of 87%, 72%, and 79%, respectively). Within eudicots, *Nelumbo*, Trochodendrales (W), and *Pachysandra* are paraphyletic with respect to the core eudicots (X), as in the *PHYC* subtrees, whereas the ranunculid clade differs from the *PHYC* subtrees in uniting Papaverales (V) with Lardizabalaceae (U), as in the *PHYA* subtrees (cf. figs. 1, 2).

The heuristic search using GeneTree (Page 1998) to infer optimal species trees from the most parsimonious gene network depicted in figure 1 retained 64 trees, with a cost of 66 (14 duplications and 52 losses). The strict consensus of these trees (fig. 5) is well resolved, and in contrast to results from combined analyses that included *Ceratophyllum*, this consensus is highly congruent with the gene subtrees (e.g., fig. 1). *Amborella* is sister to the rest of the angiosperms, and Nymphaeales diverge next from the remaining angiosperms, followed by a clade of *Austrobaileya* + *Illicium* with *Ceratophyllum*. The remaining angiosperms occur in three major clades, a clade of eudicots (G), a clade of monocots (F), and a clade of magnoliids, in which Chloranthaceae (E) are sister to Magnoliales + Laurales (D) and winteroids (J) are sister to Piperales (H). Moreover, whereas the three clades are unresolved relative to one another, relationships within them are generally well resolved and are consistent with results from other data. In GeneTree analyses, the two gene subtrees are reconciled by inferring that a number of undetected duplica-

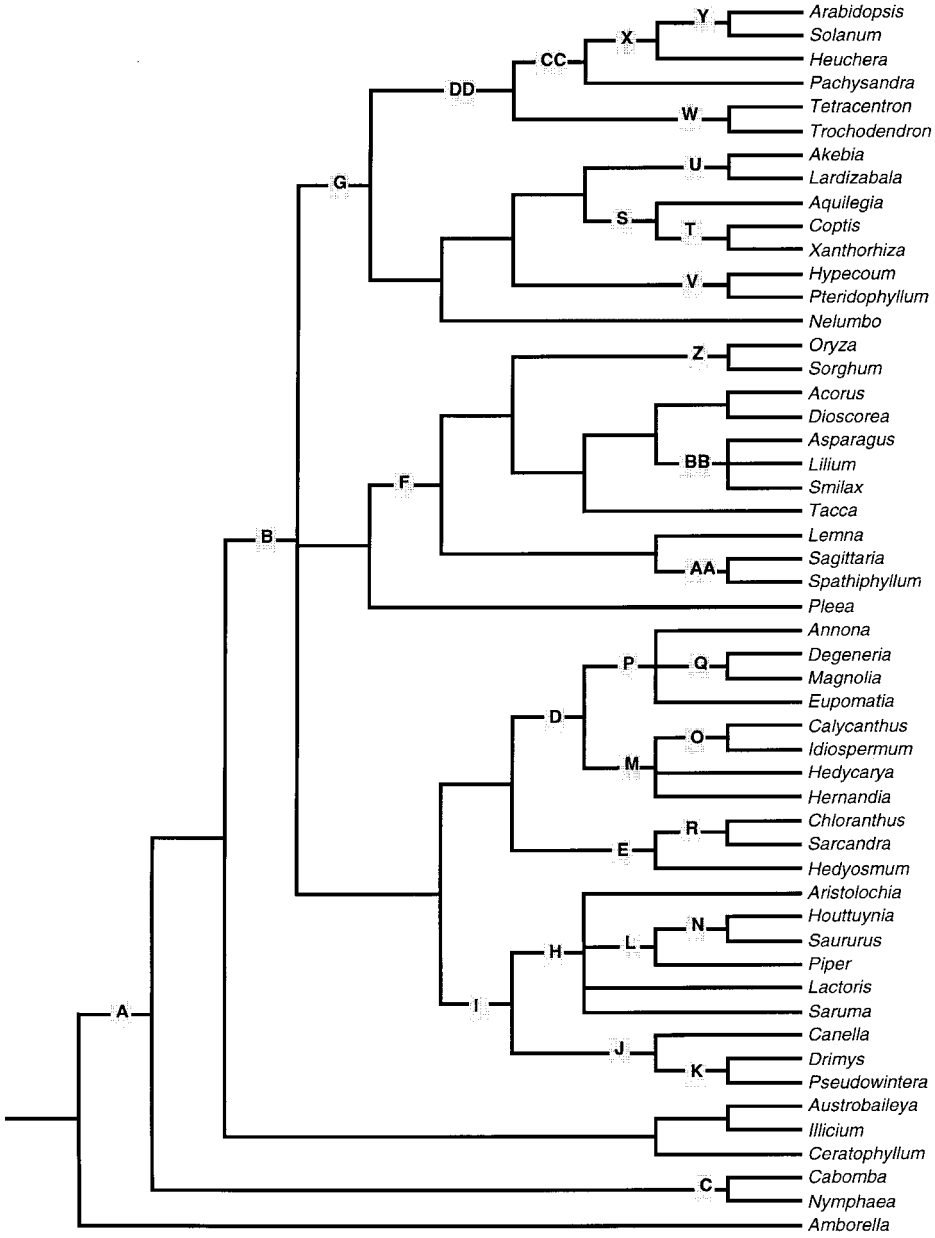


Fig. 5 Strict consensus of 64 most parsimonious rooted species trees resulting from analysis of the gene network in figure 1 by using gene tree parsimony implemented with GeneTree (Page 1998), minimizing duplications and losses (cost = 14 duplications and 52 losses).

tions gave rise to paralogues that were not sampled or were lost (e.g., Page 1994). However, a number of conflicts between the *PHYA* and *PHYC* subtrees are not well supported (see above) and likely result from causes other than undetected paralogy.

Discussion

The analyses presented here of *PHYA* and *PHYC* from many more taxa uphold most of the conclusions of our earlier phytochrome analyses and provide some additional insights. *Amborella* still appears to be the sister group of all other angiosperms. Notably, however, we cannot reject alternative rootings in which water lilies, either alone or in combination with *Amborella*, are basal. *Austrobaileya* still appears to be the sister group of the remaining angiosperms and is united with the newly sampled *Illicium*. Within the remaining angiosperms, eudicots and monocots form rather well-supported clades, as do Magnoliales, Laurales, Piperales, and winteroids. We find some support for a clade within which Laurales are united with Magnoliales and Piperales with winteroids. Interestingly, *Aristolochia* and *Saruma* are not united in the phytochrome trees, and in our larger analysis, at least in *PHYA* subtrees, monocots diverge first from the remaining angiosperms, followed by Chloranthaceae. Within eudicots we find support for a basal split between ranunculids (Ranunculales, Papaverales) and the rest, though the position of *Nelumbo* remains uncertain. *Acorus* is not sister to the rest of the monocots, as it is in some plastid phylogenies (Duvall et al. 1993).

The use of duplicated phytochrome genes to infer angiosperm phylogeny is an important complement to recent analyses based on nuclear ribosomal and organellar DNA sequences. We have sampled protein coding nuclear DNA sequences, and our analyses do not depend on the inclusion of divergent outgroup sequences. The relationships at the base of the angiosperms that we describe above are basically the same as those resolved in analyses (with outgroups) of data combined from plastid, mitochondrial, and nuclear ribosomal genes (Parkinson et al. 1999; Qiu et al. 1999). Moreover, many of the relationships among the remaining angiosperms that we find are the same. Unfortunately, as in Qiu et al. (1999), the positions of eudicots, magnoliids, Chloranthaceae, and monocots are not well supported (bootstrap values <50%). Similarly, the position of *Ceratophyllum* remains equivocal. Our results imply that it diverged early rather than with monocots (as in Qiu et al. 1999), but neither position is well supported.

Duplicate gene rooting, which seldom has been employed when outgroups are available (but see Sang et al. 1997; Telford and Holland 1997), is likely to prove useful elsewhere. Single-copy nuclear genes appear to be rare (Page 2000), and gene duplications may have occurred along many branches of interest. However, to make the most of gene duplications, more attention to analytical issues is needed. Our previous discussion of duplicate gene rooting distinguished between a "reciprocal outgroups" and a "minimum events" interpretation (Donoghue and Mathews 1998). Under the reciprocal outgroups view (see Doolittle and Brown 1994), sequences of one of the gene copies are viewed as outgroups for the other, and vice versa, and a rooted species tree is derived by consensus of the two gene subtrees. In contrast, under the minimum events view, the best-rooted species tree is the one that minimizes additional duplications and losses, lineage sorting, and lateral transfer events in the gene tree (e.g., Donoghue and Mathews 1998, fig. 2; see discussion of gene tree parsimony in Slowinski and Page 1999). In theory we prefer the minimum events interpretation, but we know of no algorithm to infer reconciled trees that deals simultaneously with the full range of events that might lead to incongruent gene trees and which also takes into account the underlying support for the gene tree. This is a difficult optimization problem, even if relative weights could be assigned beforehand to the different processes (Maddison 1997). In the meantime, to partially implement the logic of the minimum events approach, we have used Page's (1998) GeneTree program to identify species trees that minimize gene duplications and losses.

Weston (1994) provided yet another interpretation of duplicate gene rooting. This hinges on assessing the polarity of individual characters by determining the distribution of states across paralogous forms of a gene. If one nucleotide, for example, A, is universally present at a particular site in one form of the gene but there is variation at that site in the second form of the gene (e.g., A is found in some species and C in others), then the nucleotide present in both gene forms (A) is considered ancestral and the alternative state (C) derived. A polarized character implies that the root does not lie near species with the derived condition. Therefore, by assessing the polarity of each of the characters that vary across paralogous genes, it may be possible to infer a rooted species tree.

Regardless of interpretation, duplicate gene rooting allows the inference of a rooted species tree without inclusion of sequences from outgroups. Previously (Donoghue and Mathews 1998; Mathews and Donoghue 1999) we argued that this could be useful if outgroup sequences were so highly diverged from ingroup sequences that their inclusion would create long branch artifacts. In effect, a duplication occurring along the branch to the ingroup would bisect the long branch connecting the ingroup with outgroups (fig. 6*A*, 6*B*). However, as we also noted (Donoghue and Mathews 1998), the impact of this bisection will depend on where along the branch the duplication occurred and on how rapidly the genes have evolved (fig. 6*B*–6*F*). If sequences evolve in a clocklike manner, the branch between two paralogues will always be shorter than the branch between a paralogue and an outgroup, though the difference may be slight depending on the time elapsed before the duplication (fig. 6*B*, 6*C*). In contrast, if evolution is not clocklike, there may be cases in which the branch that separates paralogues would be longer than the branch between one or both of the paralogues and the outgroup sequences (fig. 6*D*). Even when rates of evolution differ between paralogues, these rates still may be less diverged from one another than from outgroup sequences (fig. 6*E*, 6*F*). Clearly, the impact of excluding outgroups from analyses of duplicate genes is a complex function of when the duplication occurred and the rates of evolution. It is unclear at this time which of the patterns depicted in figure 6 will be encountered most often (cf. Li and Gojobori 1983; Goodman et al. 1987; Iwabe et al. 1996; Cronn et al. 1999). But as the phytochrome analyses show, even when rates of divergence are high, duplicate gene rooting can provide valuable insight.

We also noted previously the need for more attention to be

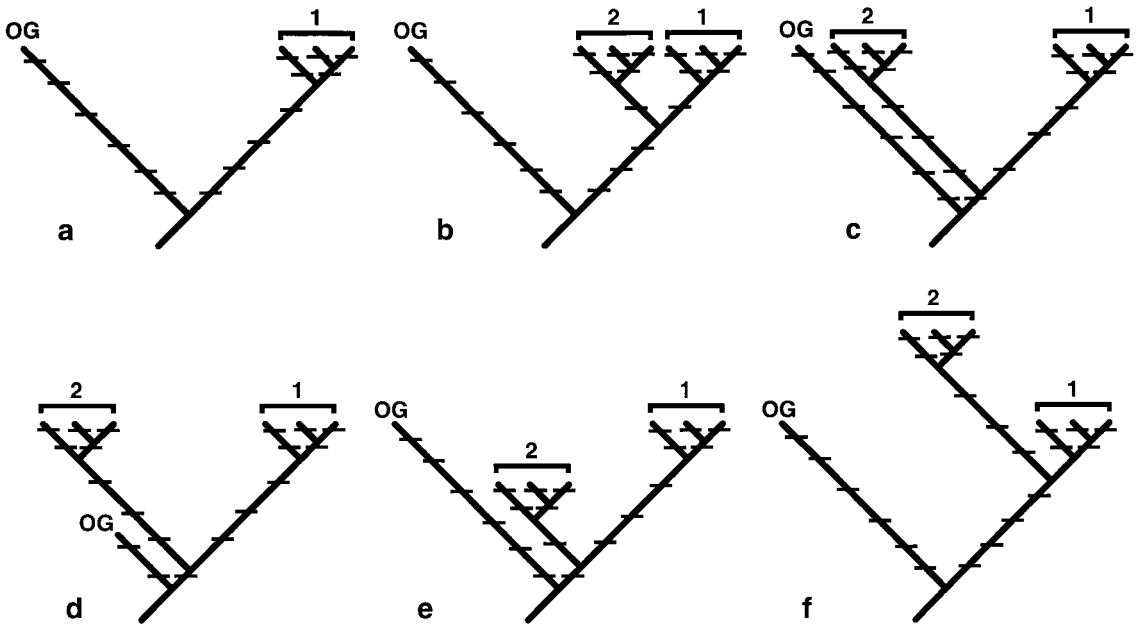


Fig. 6 The effect of duplication time and rates of nucleotide evolution on branch lengths between a pair of duplicated genes and outgroup sequences. Hatch marks indicate numbers of changes along branches (*OG* = outgroups; *1* = paralogue 1; *2* = paralogue 2). *a*, Rooted tree of one outgroup sequence and three sequences of one of the duplicated genes. *b–f*, Rooted trees of one outgroup sequence and six paralogous ingroup sequences resulting from a late duplication when evolution is clocklike (*b*), an early duplication when evolution is clocklike (*c*), an early duplication when evolution of the outgroup is slow relative to the paralogues (*d*), an early duplication when evolution of one paralogue is slow relative to the other paralogue and the outgroup (*e*), and a late duplication when the rate of one paralogue is high relative to the other paralogue and the outgroup (*f*).

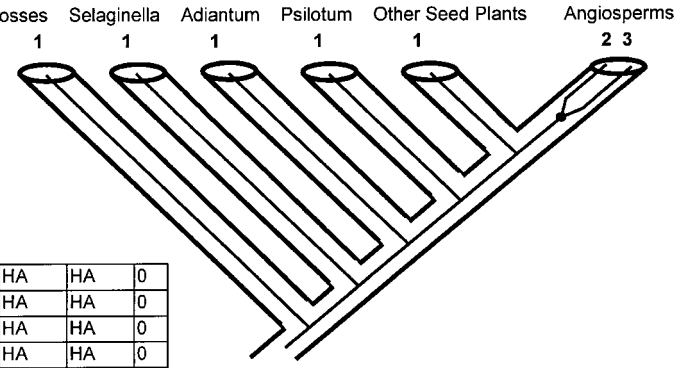
paid to the treatment of unduplicated outgroup sequences in analyses of duplicated genes to obtain a rooted species tree (Donoghue and Mathews 1998). Simmons et al. (2000) proposed "uninode coding" to combine unduplicated and duplicated sequences in a matrix in which species are terminals. After an initial analysis of all gene copies to establish the phylogenetic position of gene duplications, hypothetical ancestral sequences are reconstructed, each corresponding to ancestors at inferred duplications. A matrix is then constructed in which each species is scored for each form of the gene. A single duplication yields three forms of a gene—the unduplicated form and the two duplicated forms. Species with an unduplicated form are coded as having the hypothetical ancestral sequence for the two duplicated forms of the gene (fig. 7*A*). Species with two forms are coded as having the hypothetical ancestral sequence for the unduplicated form of the gene. A single binary character is added to reflect the duplication event, and the matrix is analyzed to obtain a species tree.

Uninode coding is designed to resolve the issue of including outgroups with unduplicated sequences when duplicate genes are combined for analysis. But in some cases it will not be appropriate, and in others it may not be the best of the available approaches. Simmons et al. (2000) noted that uninode coding should not be used if duplicate loci are subject to concerted evolution. Similarly, it seems inappropriate when other processes, such as duplication and loss, lineage sorting, and lateral transfer, have caused genuine and strong conflicts between duplicate gene trees. In these cases, which may be common in multigene families (Morton et al. 1996; Clegg et al. 1997), approaches that do not involve data combination may be more appropriate (see de Quieroz et al. 1995). In other cases, analyses that exclude outgroups will be useful for comparison with results from analyses in which artifacts are suspected to result from long branches (Felsenstein 1978; Kim 1996) that lead to outgroups.

In cases in which uninode coding does seem appropriate, there are other concerns. First, because it is a sequential procedure, final estimation of the species phylogeny may be based on erroneous assumptions (e.g., Maddison et al. 1984; Nixon and Carpenter 1993). For example, if the initial gene tree is not well supported and/or the gene family is not well sampled, a uninode analysis may be built on erroneous inferences about hypothetical ancestors. Second, the use of hypothetical ancestors increases the number of polymorphic characters and taxa in uninode matrices, which may sometimes be problematic (e.g., Nixon and Davis 1991).

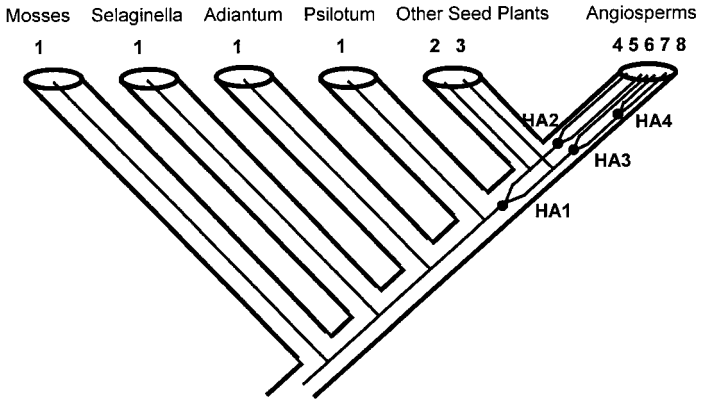
There also are significant challenges involved in the construction of a uninode matrix. Simmons et al. (2000) asserted that the method could be generalized to cases in which there are multiple duplications, but their reanalysis of phytochrome data from Donoghue and Mathews (1998) failed to demonstrate this. Our analysis provided evidence of four gene duplications (Donoghue and Mathews 1998, fig. 3), as illustrated by the gene tree in figure 7*B*. Simmons et al. (2000) based their uninode analysis on just part of our tree, ignoring duplications leading to the two forms in seed plants and the additional forms within angiosperms (cf. Donoghue and Mathews 1998, fig. 3; Simmons et al. 2000, fig. 1*b*). The sequences

a



Mosses	PHY1	HA	HA	0
Selaginella	PHY1	HA	HA	0
Adiantum	PHY1	HA	HA	0
Psilotum	PHY1	HA	HA	0
Other Seed	PHY1	HA	HA	0
Angiosperms	HA	PHY2	PHY3	1

b



Mosses	PHY1	HA1	HA1	HA2	HA2	HA3	HA4	HA4
Selaginella	PHY1	HA1	HA1	HA2	HA2	HA3	HA4	HA4
Adiantum	PHY1	HA1	HA1	HA2	HA2	HA3	HA4	HA4
Psilotum	PHY1	HA1	HA1	HA2	HA2	HA3	HA4	HA4
Other Seed	HA1	PHY2	PHY3	HA2	HA2	HA3	HA4	HA4
Angiosperms	HA1	HA?	HA?	PHY4	PHY5	PHY6	PHY7	PHY8

Fig. 7 Schematic representations of gene phylogenies within their containing species phylogenies and their corresponding uninode matrices. Filled circles at duplication points in the gene phylogenies represent hypothetical ancestors (*HA*) of two descendant gene lineages. *a*, Summary of phytochrome gene phylogeny presented by Simmons et al. (2000), showing only a single gene duplication. *b*, Summary of the phytochrome gene phylogeny presented by Donoghue and Mathews (1998), showing four gene duplications. Characters to reflect gene duplication events (Simmons et al. 2000) are omitted from the corresponding matrix shown here.

excluded from their analysis (e.g., *PHYB*, *PHYD*, and *PHYE*) might bear significantly on the reconstruction of hypothetical ancestors. Furthermore, Simmons et al. (2000) did not precisely indicate how species should be coded when a series of gene duplications are nested within one another (e.g., how angiosperms should be scored for PHY2 and PHY3 in fig. 7*B*). Reanalysis of the data in Donoghue and Mathews (1998, fig. 3) should have included four hypothetical ancestors and eight gene forms (fig. 7*B*). A uninode matrix for the phytochrome sequences now available in GenBank would include at least 11 forms of the gene, since at a minimum, there are two copies in *Adiantum*, three in nonflowering seed plants, and three to five copies in angiosperms. In view of the uncertainty surrounding uninode coding when duplications are nested within one another, we have not presented a uninode analysis of our present data set. However, just as the uninode results of Simmons et al. (2000) were congruent with our previous analyses, our experiments with uninode coding have yielded results that are similar in all major respects to the results we report here based on combined analysis of *PHYA* and *PHYC* and on reconciled trees that minimize gene duplications and losses.

Conclusions

Our results provide insights into relationships at the base of the angiosperm tree and are an important complement to

recent analyses based on nuclear ribosomal and organellar DNA sequences. They also imply that there may be diminishing returns in much more intensive sampling of *PHYA* and *PHYC* to resolve relationships among monocots, magnoliids, Chloranthaceae, *Ceratophyllum*, and eudicots. Instead, we expect phytochrome sequences to provide convincing resolution of more recent divergences (e.g., Mathews and Sharrock 1996; Lavin et al. 1998; Mathews et al. 2000; Simmons et al., in press).

Our results also highlight the use of duplicate gene rooting, which is likely to prove useful elsewhere. Within the phytochrome gene family, other duplications may be useful for resolving relationships among seed plant lineages and within eudicots. But as we noted, more attention to analytical issues is needed in order to make the most of gene duplications in this way. Specifically, methods are needed to infer reconciled trees that simultaneously consider several different processes leading to incongruence (e.g., gene duplication and loss, lineage sorting) and that take into account the possibility that the underlying gene trees may be wrong or only weakly supported.

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