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Phylogenetic relationships among early-diverging eudicots based on four genes: were the eudicots ancestrally woody?

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Abstract

Based on analyses of combined data sets of three genes (18S rDNA, *rbcL*, and *atpB*), phylogenetic relationships among the early-diverging eudicot lineages (Ranunculales, Proteales, Trochodendraceae, Sabiaceae, and Buxaceae) remain unclear, as are relationships within Ranunculales, especially the placement of Eupteleaceae. To clarify relationships among these early-diverging eudicot lineages, we added entire sequences of 26S rDNA to the existing three-gene data set. In the combined analyses of four genes based on parsimony, ML, and Bayesian analysis, Ranunculales are strongly supported as a clade and are sister to other eudicots. Proteales appear as sister to the remaining eudicots, which are weakly (59%) supported as a clade. Relationships among Trochodendraceae, Buxaceae (including *Didymeles*), Sabiaceae, and Proteales remain unclear. Within Ranunculales, Eupteleaceae are sister to all other Ranunculales, with bootstrap support of 70% in parsimony analysis and with posterior probability of 1.00 in Bayesian analysis. Our character reconstructions indicate that the woody habit is ancestral, not only for the basal angiosperms, but also for the eudicots. Furthermore, Ranunculales may not be ancestrally herbaceous, as long maintained. The woody habit appears to have been ancestral for several major clades of eudicots, including Caryophyllales, and asterids.

Keywords: 26S rDNA; Early-diverging eudicots; *Euptelea*; Ranunculales; Woodiness

1. Introduction

Recent molecular phylogenetic studies of angiosperms have consistently recognized the eudicot clade, members of which have triaperturate or triaperturate-derived pollen as a morphological synapomorphy (e.g., Albert et al., 1998; Chase et al., 1993; Drinnan et al., 1994; Hoot et al., 1999; Soltis et al., 1997, 2000; Soltis et al., 1999). The eudicots contain 75% of all angiosperm species (Drinnan et al., 1994; Mabberley, 1987; Magallón et al., 1999). In the eudicot clade, the early-diverging, or basal, eudicots, i.e., Ranunculales, Proteales, Sabiaceae, Trochodendraceae, and Buxaceae, are followed by a well-supported clade of core eudicots, consisting of the asterids, rosids, Caryophyllales, Gunnerales, Berberidopsidales (Berberidopsidaceae/Aextoxicaceae), Santalales, and Saxifragales.

In a previous multi-gene study, Hoot et al. (1999) analyzed 73 taxa including early-diverging eudicots, basal angiosperm outgroups, and placeholders for core eudicots using two chloroplast genes (*rbcL* and *atpB*) and the nuclear 18S rDNA (Fig. 1A). They found that Ranunculales were sister to all other eudicots; Proteales (including Platanaceae and Nelumbonaceae), Sabiaceae, Buxaceae (including *Didymeles*), and Trochodendraceae (including *Tetracentron*) were then successive sister groups to the core eudicots. However, most of these relationships did not receive bootstrap support >50%.

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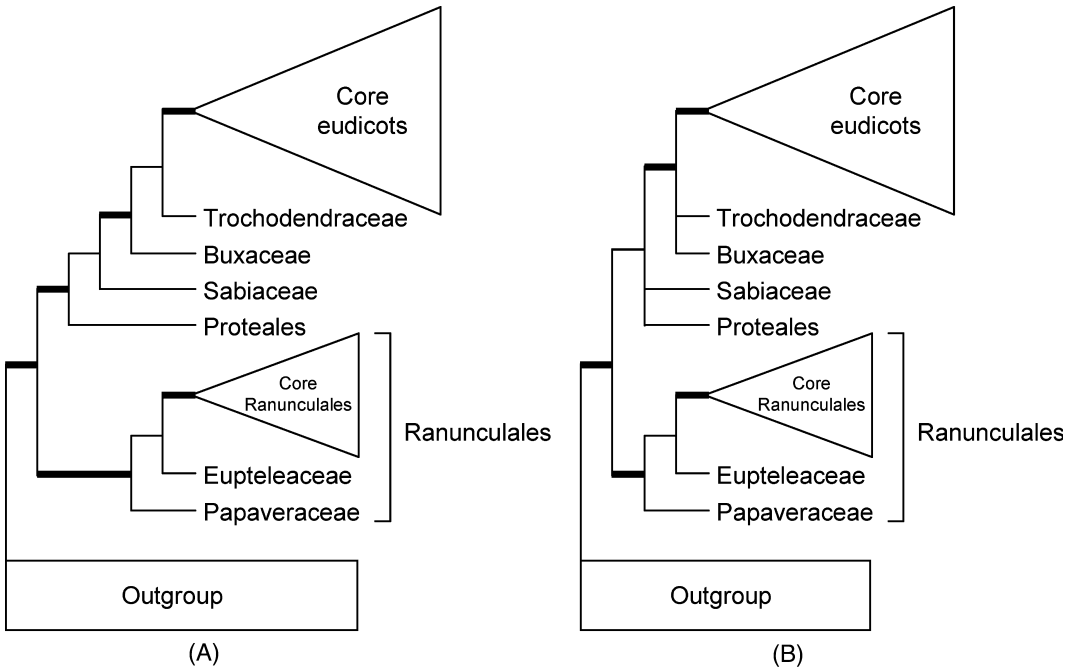

Fig. 1. Summary trees based on previous molecular phylogenetic studies of early-diverging eudicots. (A) Strict consensus tree from Hoot et al. (1999). (B) Jackknife consensus tree from Soltis et al. (2000). Bold lines indicate clades receiving support >70%.

Within Ranunculales, they found Papaveraceae to be sister to Eupteleaceae + a strongly supported core Ranunculales (Berberidaceae, Circaeasteraceae, Lardizabalaceae, Menispermaceae, and Ranunculaceae). However, bootstrap support of this sister group relationship was >50%.

Soltis et al. (2000) expanded the taxon sampling substantially in an analysis of three-genes (*rbcL*, *atpB*, and 18S rDNA) for 560 angiosperm species. In that study, the relationships among early-diverging eudicots were consistent with those of Hoot et al. (1999), despite of somewhat different taxon sampling (Fig. 1B). Although previous studies (Hoot et al., 1999; Soltis et al., 2000) provided insights into relationships among some groups of early-diverging eudicots, many problems still remain in our understanding of phylogenetic relationships among these plants. Importantly, reconstructing patterns of character evolution in the eudicots as a whole requires resolution of relationships among early-diverging eudicots.

The 18S ribosomal RNA gene has been the most widely used nuclear sequence for phylogeny reconstruction at higher taxonomic levels in plants (e.g., Chaw et al., 1997; Hamby and Zimmer, 1992; Soltis et al., 1997). However, 18S rDNA data alone may provide too few phylogenetically informative characters to resolve relationships adequately, even among families of angiosperms (Soltis and Soltis, 1998). Kuzoff et al. (1998) demonstrated the potential of entire 26S rDNA sequences for phylogeny reconstruction at taxonomic levels comparable to those investigated with 18S rDNA. They provided a protocol for PCR amplification and sequencing of entire (~3.4 kb) 26S rDNA sequences as single amplicons and primers that can be used for amplification and sequencing. 26S rDNA evolves 1.6–2.2 times faster than and provides 3.3 times as many parsimony-informative characters as 18S rDNA, and the expansion segments of 26S rDNA evolve 1.2 to 3.0 times faster than *rbcL*, providing 1.5 times the number of informative characters (Kuzoff et al., 1998). The phylogenetic utility of 26S rDNA sequences in angiosperms has been further demonstrated by several recent studies (e.g., Fan and Xiang, 2001; Fishbein et al., 2001; Zanis et al., 2002, 2003).

Phylogenetic analyses have revealed that Ranunculales not only form a well-supported clade (with the addition of the woody family Eupteleaceae, traditionally placed in Hamamelidae), but also they occupy a pivotal phylogenetic position as sister to all other eudicots (Hoot et al., 1999; Soltis et al., 2000). There has been considerable debate concerning the primitive or ancestral habit in the angiosperms, woody vs. herbaceous. Authors of modern classifications (e.g., Cronquist, 1981; Takhtajan, 1997) favored a woody ancestral condition. However, early cladistic analyses placed herbaceous taxa (e.g., Chloranthaceae) as sister to all other angiosperms (Donoghue and Doyle, 1989). Early molecular studies similarly placed herbaceous taxa (e.g., Nymphaeaceae or Ceratophyllaceae) as sister to other angiosperms (e.g., Chase et al., 1993; Doyle et al., 1994), whereas recent analyses have converged on *Amborella* (woody) followed by Nymphaeaceae (herbaceous) as

subsequent sisters to all other angiosperms (see Hilu et al., in press; Kim et al., submitted; Zanis et al., 2002, 2003). However, some of the earliest fossil angiosperms are herbaceous aquatics, including *Archaefructus* (Friis et al., 2001; Sun et al., 2002). Character reconstructions generally show the ancestral condition for angiosperms to be ambiguous (Zanis et al., 2003). Within the eudicots, however, Ranunculales are primarily herbaceous, leading to Cronquist's (1968) characterization of them as "the herbaceous equivalent of the Magnoliales." The position of Ranunculales as sister to all other eudicots, raises the possibility that the eudicots might be ancestrally herbaceous.

In this paper, we present the results of phylogenetic analyses of early-diverging eudicots based on combined sequences for four genes, *rbcL*, *atpB*, 18S rDNA, and 26S rDNA. Our goal was to clarify relationships among the early-diverging eudicots in general, and within Ranunculales in particular. Elucidation of phylogenetic relationships among clades of early-diverging eudicots provides the opportunity to assess character evolution. We reconstructed the evolution of one important trait, the woody habit.

2. Materials and methods

2.1. Taxon sampling

Fifty-three taxa representing the major lineages of early-diverging (29 taxa) and core eudicots (24 taxa), plus seven basal angiosperm outgroups (one representative each of Magnoliaceae, Himantandraceae, Winteraceae, Chloranthaceae, Aristolochiaceae, Nymphaeaceae, and Amborellaceae) were included in this study. All early-diverging eudicot lineages were represented: Buxaceae, Sabiaceae, Trochodendraceae, all three families of Proteales (Proteaceae, Platanaceae, and Nelumbonaceae), and all seven families of Ranunculales (Lardizabalaceae, Circaeasteraceae, Menispermaceae, Berberidaceae, Ranunculaceae, Papaveraceae, and Eupteleaceae). Outgroup taxa and placeholders for core eudicots were chosen from the three-gene analysis of angiosperms (Soltis et al., 1999, 2000) and the APG (1998; APG II, 2003) classifications (Table 1). Sequences of *rbcL*, *atpB*, and 18S rDNA were reported previously (Hoot et al., 1999; Soltis et al., 2000), as were some of the 26S rDNA sequences (Fishbein et al., 2001; Kuzoff et al., 1998; Zanis et al., 2003). We generated 26S rDNA sequences for 38 taxa (Table 1). Whenever possible, 26S rDNA was sequenced from the same DNA sample used in previous studies (e.g., Hoot et al., 1999; Soltis et al., 2000). However, in a few cases, we used DNA from another species of the same genus, or another genus of the same family as a placeholder (Table 1). Instances in which a different genus was used are: *Jepsonia* for 26S rDNA, *Boykinia* for three-gene; *Fragaria* for 26S rDNA, *Kerria* for three-gene; and *Lambertia* for 26S rDNA, *Placospermun* for three-gene. We followed the APG II (2003) treatment for familial and ordinal circumscriptions and names.

2.2. Molecular methods

For most taxa, 26S rDNA was amplified as two fragments from total DNA extracts. The PCR primers used were: N-nc26S1 (forward) and 1839rev (reverse), and N-nc26S7 (forward) and 3331rev (reverse), respectively (Kuzoff et al., 1998). Methods of amplification and sequencing followed Kuzoff et al. (1998). Automated sequencing of purified PCR products was conducted on an ABI 377 Automated Sequencer (Applied Biosystems, Foster City, CA). A subset of the primers used by Kuzoff et al. (1998) was sufficient to obtain complete 26S rDNA sequences in most cases: N-nc26S1, N-nc26S3, N-nc26S5, N-nc26S7, N-nc26S9, N-nc26S11, N-nc26S13, 268rev, 641rev, 950rev, 1229rev, 1449rev, 1839rev, 2426rev, 2782rev, and 3331rev. However, for *Buxus sempervirens* L. (Buxaceae) and *Viscum album* L. (Santalaceae), we were unable to obtain approximately 100 and 280 bp, respectively, of the 26S rDNA region. These areas were coded as missing characters. Proofreading and editing of each sequence were performed using Sequencher version 3.0 (Gene Codes, Ann Arbor, Michigan, USA). Sequences were aligned using CLUSTAL X (Thompson et al., 1997), and then adjusted by eye.

2.3. Phylogenetic analyses

Phylogenetic analyses were performed separately on the individual *rbcL*, *atpB*, 18S rDNA, and 26S rDNA data sets as well as on several combined data sets: *rbcL* + *atpB* (chloroplast genes), 18S rDNA + 26S rDNA (nuclear genes), and *rbcL* + *atpB* + 18S rDNA + 26S rDNA (total evidence). To ascertain the impact of the addition of 26S rDNA sequences and to facilitate direct comparison with the three-gene eudicot analysis of Hoot et al. (1999), we also examined a parallel three-gene data set (*rbcL*/*atpB*/18S rDNA) of identical taxon composition to the four-gene data set, but from which the 26S rDNA sequences were removed.

Data incongruence among the four genes was explored using the partition homogeneity test (= incongruence length difference test of Farris et al., 1995) implemented in PAUP* 4.0b10 (Swofford, 1998). This test employed 100 replicates, each with 10 random addition replicates using NNI branch swapping and saving a maximum of 5000 trees per replicate.

All parsimony analyses were performed using PAUP* 4.0b10 (Swofford, 1998) using the following

Table 1
Species analyzed in this study. Species are arranged by families and higher groups according to the APG II (2003) system

Family	Species	GenBank Accession Number			
		26S rDNA/voucher information	*atpB*	*rbcL*	18S rDNA
Amborellaceae	*Amborella trichopoda* Baill.	AF479238	AJ235389	L12628	U42497
Chloranthaceae	*Chloranthus japonicus* Siebold	AF479245	AJ235431	L12640	
	Chloranthus multistachys S. J. Pei				AF206885
Nymphaeaceae	*Cabomba caroliniana* A. Gray	AF479239	AF209549	M77027	AF206878
Canellales					
Winteraceae	*Drimys winteri* J. R. Foster & G. Foster	AF036491	AF093425	L01905	U42823
Magnoliales					
Himantandraceae	*Galbulimima belgraveana* Sprague	**AF389251**/*Qiu 90034 NCU*	AJ235478	L12646	AF206916
Magnoliaceae	*Magnolia denudata* Desr.	**AF389256**/*S. Kim 1010 NPRI*			
	Magnolia tripetala L.		AJ235526	AJ131927	AF206956
Piperales					
Aristolochiaceae	*Aristolochia macrophylla* Lam.	AY095450	AJ235399	L12630	AF206855
EUDICOTS					
Buxaceae	*Buxus sempervirens* L.	**AF389243**/*Chase 203 NCU*	AF092110	AF093717	L54065
	Pachysandra procumbens Michx.	**AF389244**/*Chase 207 NCU*	AF092111	AF093718	AF094533
	Didymeles perrieri Leandri	**AF389247**/*Andrianantoanina 387 MO*	AF092119	AF061994	AF094541
Sabiaceae	*Meliosma vetichiorum* Hemsl.	**AF389271**/*Chase 2989 K*	AF209629	AF206793	AF206951
	Sabia swinhoei Hemsl. ex F. B. Forbes & Hemsl.	**AF389272**/*Wagner 6518 HAST*	AF093395		L75840
	Sabia sp.			L12662	
Trochodendraceae	*Tetracentron sinensis* Oliv.	AF274670	AF093422	L12668	U42814
	Trochodendron aralioides Siebold & Zucc.	AF274671	AF093423	L01958	U42816
Proteales					
Nelumbonaceae	*Nelumbo lutea* (Willd.) Pers.	**AF389259**/*Hoot 9212 UWM*	AF093387	M77032	L75835
Platanaceae	*Platanus occidentalis* L.	AF274662	U86386	L01943	U42794
Proteaceae	*Roupala macrophylla* Pohl	**AF389265**/*Douglas 131 MEL*	AF060416	AF093728	AF094559
	Lambertia inermis R. Br.	AF274652			
	Placospermum coriaceum C. T. White & W. D. Francis		AF060391	AF093729	L75837
Ranunculales					
Berberidaceae	*Caulophyllum thalictroides* (L.) Michx.	**AF389240**/*Hoot 925 UWM*	AF092108	L08760	L54064
	Nandina domestica Thunb.	**AF389241**/*Hoot 922 UWM*	L37930	L37920	L37911
Circaeasteraceae	*Circaeaster agrestis* Maxim.	**AF389246**/*Chase 506 K*	AF092116	AF093720	AF094538
	Kingdonia uniflora Balf. f. & W. W. Sm.	**AF389245**/*Qiu s.n. PE*	AF092115	AF093719	AF094537
Eupteleaceae	*Euptelea polyandra* Siebold & Zucc.	**AF389249**/*Qiu 90026 NCU*	U86384	L12645	L75831
Lardizabalaceae	*Akebia quinata* (Houtt.) Decne.	**AF389253**/*Qiu 91020 NCU*	L37924	L12627	L31795
	Decaisnea fargesii Franch.	**AF389254**/*Reznicek 9236 MICH*	L37926	L37916	L37907
	Sinofranchetia chinensis Helmsl.	**AF389255**/*Edinburgh 831635 F*	L37931	L37921	L37912
Menispermaceae	*Menispermum canadense* L.	**AF389257**/*Soltis & Soltis 2526 WS*	AF093384	AF093726	L75834
	Tinospora caffra Miers	**AF389258**/*Jaarsveld 2131 NBG*	L37933	L37923	L37914
Papaveraceae	*Dicentra eximia* Torrey	**AF389262**/*Reznicek 9756 MICH*			L37908
	Dicentra chrysantha Walp.		AJ235454		
	Dicentra spectabilis (L.) Lem.			L08761	
	Hypecoum imberbe Sm.	**AF389263**/*Chase 528 K*	U86398	U86628	L75836
	Pteridophyllum racemosum Siebold & Zucc.	**AF389264**/*Chase 531 K*	U86400	U86631	AF094560
Ranunculaceae	*Coptis trifolia* (L.) Salisb.	**AF389266**/*Voss & Howard s.n. MICH*	AF093393	AF093730	L75838
	Glaucidium palmatum Siebold & Zucc.	**AF389267**/*Hoot 924 UWM*	AF093375	AF093723	L75829
	Hydrastis canadense L.	**AF389268**/*Naczi 2883 MICH*	AF093382	L75849	L75828

Table 1 (*continued*)

Family	Species	GenBank Accession Number			
		26S rDNA/voucher information	*atpB*	*rbcL*	18S rDNA
	Ranunculus keniensis Milne-Redhead & Turrill	**AF389269**/*Chase 573 K*			
	Ranunculus sp.				
	Ranunculus sardous Crantz				L24092
	Ranunculus trichophyllus Chaix			L08766	
	Xanthorhiza simplicissima Marshall	**AF389270**/*Qiu 91030 NCU*	AF093394	L12669	L75839
CORE EUDICOTS					
Aextoxicaceae	*Aextoxicon punctatum* Ruiz & Pav.	**AF389239**/*Chase 959 K*	AJ235384	X83986	AF206839
Berberidopsidaceae	*Berberidopsis corallina* Hook.	**AF389242**/*Chase 555 K*	AJ235409	AJ235773	AF206866
Gunnerales					
Gunneraceae	*Gunnera manicata* Linden	**AF389250**/*Kurcheberg s.n. WTU*		L11186	U43787
	Gunnera hamiltonii Kirk. ex W. S. Ham.		AF093374		
Caryophyllales					
Droseraceae	*Drosera capensis* L.	**AF389248**/*Chase 2582 K*			U42532
	Drosera communis A. St.-Hil.		AJ235459		
	Drosera spathulata Labill.			L19530	
Nepentheaceae	*Nepenthes* sp.	**AF389260**/*Nickerent 3056 SIU*			U42787
	Nepenthes alata Blanco		AJ235542	L01936	
Plumbaginaceae	*Plumbago auriculata* Lam.	AF036492			U42795
	Plumbago capensis Thunb.			M77701	
	Plumbago zeylanica L.		AJ235565		
Santalales					
Olacaceae	*Schoepfia schreberi* J. F. Gmel.	**AF389261**/*Nickrent 2599 ILL*	AF209671	L11205	AF207017
Santalaceae	*Eubrachion ambiguum*	**AF389273**	AF209583	L26071	L24141
	(Hook. & Arnott) Engl.	*Nickrent 2699 SIU*			
	Osyris lanceolata Hochst. & Steud.	**AF389274**/*Nickrent 2731 SIU*	AF209641	L11196	U42803
	Viscum album L.	**AF389275**/*Nickrent 2253 SIU*	AF209695	L26078	U42821
Saxifragales					
Cercidiphyllaceae	*Cercidiphyllum japonicum* Siebold & Zucc.	AF274639	AF092112	L11673	U42518
Hamamelidaceae	*Hamamelis virginiana* L.	AF036495	AF093380		AF094551
	Hamamelis mollis Oliv.			L01922	
Saxifragaceae	*Boykinia intermedia* (Piper) G. N. Jones				U42806
	Boykinia rotundifolia Parry ex A. Gray		AJ235417	L11175	
	Jepsonia parryi Small	AF036497			
	Saxifraga mertensiana Bong.	AF036498	AF209669	U06216	U42811
ROSIDS					
Vitaceae	*Leea guineensis* G.Don	AF274653	AJ235520	AJ235783	AF206951
EUROSIDS I					
Celastrales					
Parnassiaceae	*Parnassia fimbriata* Banks	AF036496		L01939	U42809
	Parnassia palustris L.		AJ235552		
Oxalidales					
Cunoniaceae	*Eucryphia lucida* Druce	AF036494	AF209584	L01918	U4253
Rosales					
Rosaceae	*Fragaria* X *ananassa* Duchesne	X58118			
	Kerria japonica (Thunb.) DC.		AF132886	AF132893	AF132890

EUROSIDS II						
Brassicales						
Brassicaceae	*Brassica napus* L.	D10840	AF209545	M88342		X17062
	Brassica balearica Pers.					
	Brassica oleracea L.					
	Brassica hirta Moench					
Sapindales						
Rutaceae	*Citrus aurantium* L.					U38312
	Citrus limon (L.) Burm.f.	X05910	AJ238408	AJ238407		
	Citrus paradisi Macfad.					
ASTERIDS						
Cornales						
Hydrangeaceae	*Philadelphus lewisii* Pursh	**AF389252**/*Soltis & Soltis 2411 WS*	AJ236231	L11198		U42782
Ericales						
Sarraceniaceae	*Sarracenia purpurea* L.	**AF389276**/*Morgan s.n. WS*	AJ235594	L01952		U42804
	Sarracenia flava L.					
EUASTERIDS I						
Solanales						
Solanaceae	*Lycopersicon esculentum* Mill.	X13557	AJ236183	L14403		AJ230617
EUASTERIDS II						
Asterales						
Asteraceae	*Tragopogon dubius* Scop.	AF036489	AJ236197	L13647		U42502
	Tragopogon porrifolius L.					

Note. Bold-faced GenBank accession numbers are for 26S rDNA determined in this study. Sequences of species designated within brackets are combined in multi-gene analyse.

method. We first performed 200 random addition replicates with TBR branch swapping saving no more than 10 trees per replicate; we saved optimal trees from each replicate, even if they were not optimal over all replicates. We then used all of these trees (68 trees, scores ranging from 10,910 to 10,916) as starting trees for further analysis with TBR branch swapping and saving all trees. To assess support for each node, bootstrap analyses (Felsenstein, 1985) were performed using 500 replicate heuristic searches with 10 random taxon addition replicates and the TBR branch-swapping option. We saved a maximum of 5000 trees per bootstrap replicate.

For the combined analysis of four genes, Maximum likelihood (ML) analysis and Bayesian analysis (Huelsenbeck, 2000) were also performed. For the ML analysis, the program MODELTEST (Posada and Crandall, 1998) was used to determine the appropriate model of sequence evolution for this data set. The chosen model ($GTR + I + \Gamma$) was applied to the data matrix using PAUP* 4.0b10 (Swofford, 1998). The ML analysis was conducted using parameter values suggested in MODELTEST ($-\ln L = 64642.40$; A:C:G:T = 0.26: 0.22 :0.27:0.25; P_inv = 0.52; Shape = 0.54) and the three most parsimonious trees as starting trees in a heuristic search with TBR branch swapping.

Bayesian analysis was performed using MrBayes 2.01 (Huelsenbeck, 2000). We ran four chains of Markov Chain Monte Carlo (MCMC), sampling every 1000 generations for 1,000,000 generations, starting with a random tree. Stationarity was reached at approximately generation 40,000; thus, the first 40 trees were the "burn in" of the chain, and phylogenetic inferences are based on those trees sampled after generation 40,000.

2.4. Character-state reconstruction

The placement of Eupteleaceae obtained here (see below) has implications for the reconstruction of character evolution. Earlier reconstructions in early-diverging eudicots analyses have focused on floral features, including merosity (e.g., Albert et al., 1998; Zanis et al., 2003). We focused on a character of critical evolutionary importance that has not been recently evaluated in light of a phylogeny for basal eudicots. We examined the evolution of habit (woody vs. herbaceous) in the early diversification of the eudicots using MacClade version 3.04 (Maddison and Maddison, 1992). We scored habit as either herbaceous or woody, using data from the literature (e.g., Cronquist, 1981; Doyle and Endress, 2000). We reconstructed habit for basal, or early-diverging eudicots and also for the major clades of core eudicots (asterids, rosids, Caryophyllales + Dilleniaceae, and Saxifragales) using current topologies (e.g., Albach et al., 2001; Bremer et al., 2002; Fishbein et al., 2001; Soltis et al., 2000).

We constructed a summary topology for angiosperms based on our results and recently published topologies for basal angiosperms (Zanis et al., 2002, 2003) and core eudicots (Soltis et al., 2003). For early-diverging eudicots, we followed the strict consensus tree obtained in the present study. The sampling of early-diverging angiosperms was expanded from seven genera to 18 genera; the topology in our reconstruction is adapted from Zanis et al. (2002). Caryophyllales, Santalales, asterids, rosids, and Saxifragales were depicted as a polytomy following Hoot et al. (1999) and Soltis et al. (2000). Soltis et al. (2003) placed Gunnerales as sister to all other core eudicots, which we follow here. We first used the all most parsimonious states optimization in MacClade because the accelerated transformation (ACCTRAN) and the delayed transformation (DELTRAN) optimizations cannot be applied when a polytomy is present. We also explored the impact of alternative topologies on character-state reconstruction. For example, the strict consensus of the shortest trees from Soltis et al. (2000) resolves the relationships among the core eudicots, allowing use of ACCTRAN and DELTRAN optimizations. We also experimented with several different relationships among core eudicot lineages (this had no impact on the reconstruction). In addition to the analysis with Eupteleaceae followed by Papaveraceae as sister to other Ranunculales (see below), we also explored the alternative of Papaveraceae followed by Eupteleaceae as successive sister groups to other Ranunculales because previous three-gene studies (Hoot et al., 1999; Soltis et al., 2000) placed Papaveraceae as a sister to other Ranunculales.

3. Results and discussion

3.1. 26S rDNA sequence analysis

Several small gaps were needed to align the 26S rDNA sequences. The aligned sequences were 3480 nucleotides in length. We excluded 81 sites from our phylogenetic analyses. These excluded sites include five regions located in expansion segments that were difficult to align, as well as sites located near the 5′ and 3′ ends of the gene. Of the 3399 aligned sites included in the phylogenetic analyses, 1117 were variable, and 699 were potentially parsimony-informative. The number of potentially parsimony-informative sites in 26S rDNA alone (699) was approximately 70% of the total from the three-gene (*rbcL*, *atpB*, and 18S rDNA) data set (1007) (Table 2).

Partition homogeneity tests between 26S rDNA vs. 18S rDNA, 18S rDNA vs. *rbcL*, 18S rDNA vs. *atpB*, *rbcL* vs. *atpB*, and 18S rDNA vs. 26S rDNA/*rbcL atpB* showed congruence ($P > 0.05$). However, the 26S rDNA data set was not congruent with the *atpB* data set alone or with the combined 18S rDNA/*rbcL atpB* data set ($P < 0.05$; Table 3). These results demonstrate that 26S rDNA sequences possess different phylogenetic information compared with other data.

The results of the partition homogeneity test indicate there is some heterogeneity among the data partitions. It is not unusual to detect significant heterogeneity among

Table 3
P values from partition-homogeneity test, with 100 random-addition replications with NNI branch swapping

Data sets	*P* value
26S rDNA vs. 18S rDNA	0.25
26S rDNA vs. *rbcL*	0.01
26S rDNA vs. *atpB*	0.05
18S rDNA vs. *rbcL*	0.08
18S rDNA vs. *atpB*	0.13
rbcL rDNA vs. *atpB*	0.12
26S rDNA vs. 18S rDNA/*rbcL atpB*	0.01
18S rDNA vs. 26S rDNA/*rbcL atpB*	0.78
rbcL vs. 26S rDNA/18S rDNA/*atpB*	0.05
atpB vs. 26S rDNA/18S rDNA/*rbcL*	0.02
Chloroplast vs. nuclear	0.01

Table 2
Comparison of metrics for the various data sets

Data set	Total length	No. variable char.	No. informative char.	No. of trees	Length of trees	CI	RI	RC
rbcL	1396	632	425	110	2490	0.38	0.44	0.17
atpB	1414	574	393	941	2130	0.40	0.50	0.20
d 18S rDNA	1723	363	189	756	1067	0.43	0.46	0.20
id 26S rDNA	3399	1117	699	10	5079	0.33	0.41	0.14
chloroplast (*rbcL* + *atpB*)	2810	1206	818	12	4648	0.39	0.47	0.18
nuclear (26S rDNA+ 18SrDNA)	5123	1480	888	2	6527	0.33	0.36	0.12
Three gene (*rbcL* + *atpB* + 26S rDNA)	4533	1569	1007	2	5756	0.39	0.50	0.18
Four gene	7933	2686	1706	3	10,910	0.36	0.43	0.16

Note. Consistency index (CI) excludes uninformative characters. RI = retention index, RC = rescaled consistency index.

data partitions using a P values of 0.05. Some authors have indicated that the partition homogeneity test is extremely conservative (Cunningham, 1997; Sullivian, 1996). Furthermore, this is not a measure of combinability. Data sets with slight heterogeneity can be readily combined as is the case here.

Parsimony analysis of the 26S rDNA data set generated 10 shortest trees each of 5079 steps (CI = 0.33, RI = 0.41). The strict consensus tree showed some differences compared with the topology reported in previous three-gene studies (Fig. 2; Hoot et al., 1999; Soltis et al., 2000). However, few of these differences received bootstrap support >50% in either tree. This result is similar to those of other comparisons of *rbcL*, *atpB*, and 18S rDNA data sets. It seems that each individual data set alone is insufficient for recognizing relationships among early-diverging eudicots. The eudicot clade received 88% bootstrap support in the 26S rDNA analysis, but the core eudicot clade did not receive support >50%. Both clades received support close to 100% in previous studies based on combined *rbcL*, *atpB*, and 18S rDNA data sets (Hoot et al., 1999; Soltis et al., 2000). Many early-diverging eudicot families were well supported: Sabiaceae, Trochodendraceae, Proteaceae, Berberidaceae, Circaeasteraceae, Lardizabalaceae, and Menispermaceae. Ranunculales, although weakly supported (53%), appeared as sister to all remaining eudicots in the strict consensus tree. Within Ranunculales,

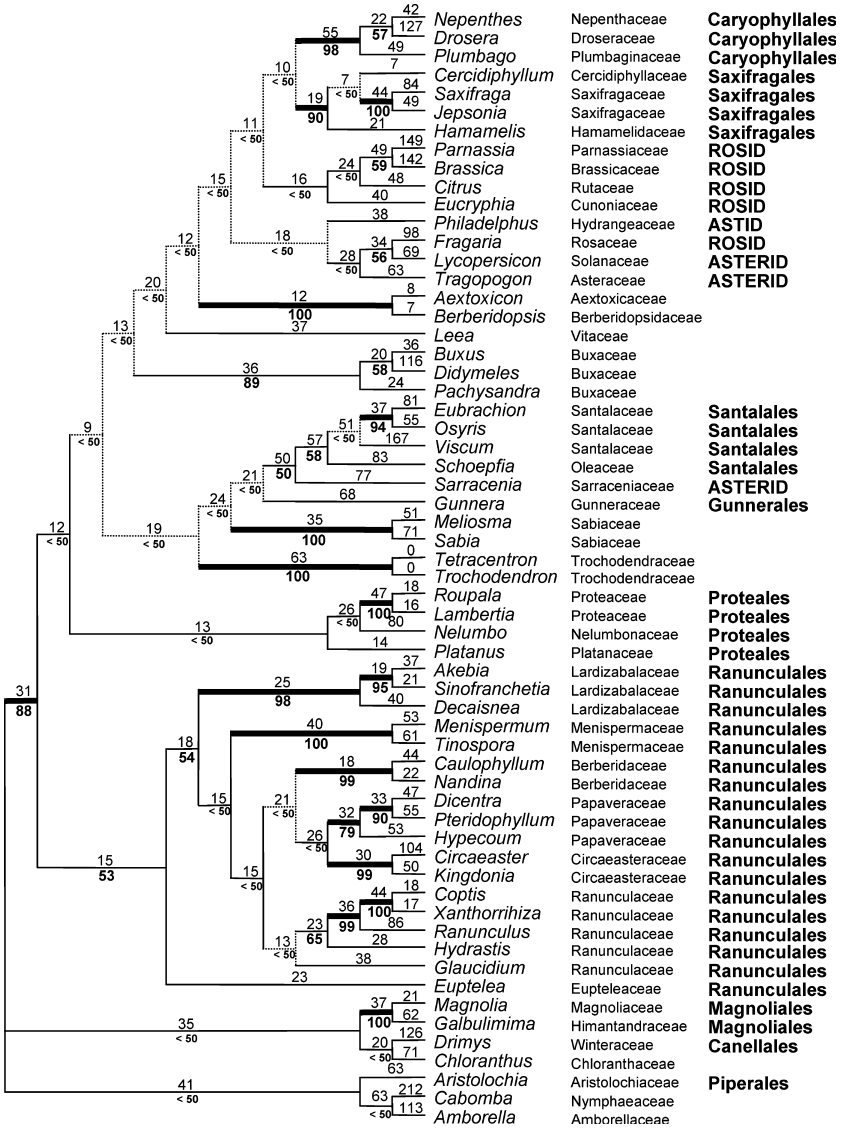

Fig. 2. One of 10 equally parsimonious trees from analysis of 26S rDNA sequences. Numbers above the branches indicate the number of nucleotide changes supporting each branch. Numbers below the branches are bootstrap percentages. Dotted lines indicate branches that collapse in the strict consensus tree. Bold lines indicate clades receiving support >70%.

Eupteleaceae are sister to the rest of the clade, although support for this sister-group relationship is low (54%). Bootstrap values for many clades within Ranunculales are >50% with 26S rDNA sequences alone.

3.2. Three-gene analysis

We conducted a three-gene analysis to determine whether our four-gene results (e.g., *Euptelea* as sister to other Ranunculales) were simply the result of the different taxon composition of our data set compared to earlier three-gene analyses (Hoot et al., 1999; Soltis et al., 2000, 2003). Two equally parsimonious trees each of 5756 steps were generated from the analysis of a combined data set of *rbcL*, *atpB*, and 18S rDNA. The only difference between this analysis and those of Hoot et al. (1999) and Soltis et al. (2000) was taxon sampling in the core eudicots and the choice of outgroup. Taxa not common to both studies were eliminated from the trees to facilitate direct comparison (Fig. 3A). Most relationships were similar. Some moderately to well-supported clades (>70%), such as eudicots, core eudicots, Ranunculales, and core eudicots + Trochodendraceae/Buxaceae, were recognized in both analyses. Our three-gene analysis also placed Papaveraceae as sister to other Ranunculales, as in Hoot et al. (1999) and Soltis et al. (2000). In the topologies of Hoot et al. (1999) and Soltis et al. (2000), the core eudicots, Tro-

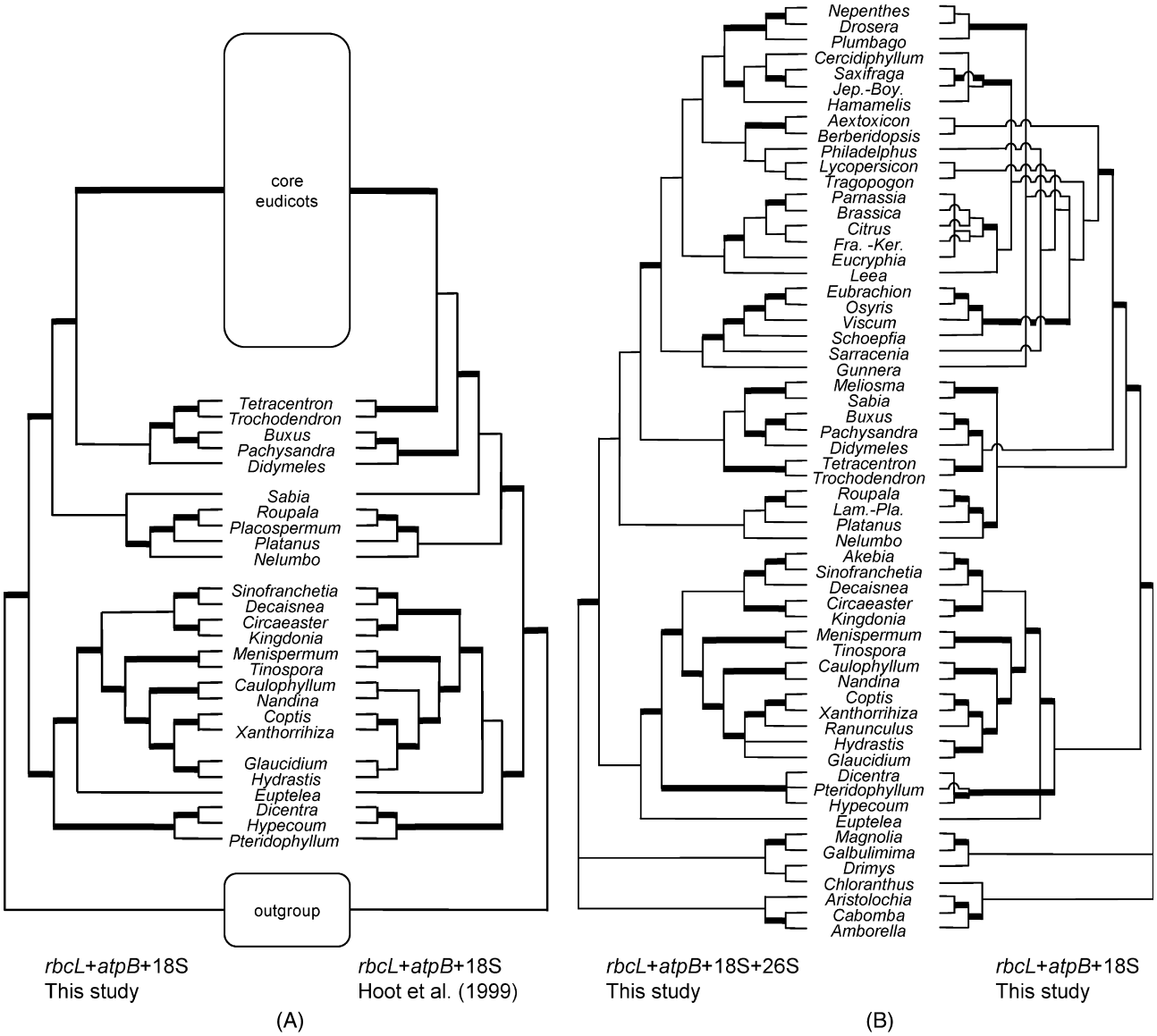

Fig. 3. Comparison of strict consensus trees from various analyses. Unshared taxa between two studies were eliminated in (A). Bold lines indicate clades supported by bootstrap values >70%.

chodendraceae, Buxaceae, Sabiaceae, Proteales, and Ranunculales form a grade. However, our three-gene analysis placed Sabiaceae as sister to Proteales and placed *Didymeles* as sister to Trochodendraceae + Buxaceae s. s. (i.e., the broadly defined Buxaceae of APG II is paraphyletic in this instance) (Fig. 3A). These unstable relationships demonstrate that relationships among early-diverging eudicots are still unclear (Hoot et al., 1999; Soltis et al., 2000).

3.3. Four-gene analysis

The number of trees obtained in the parsimony analysis of the four-gene data set was much lower than the number obtained with each gene individually (Table 3). MP analysis of the separate *rbcL*, *atpB*, 18S rDNA, and 26S rDNA data sets generated 110, 941, 756, and 10 shortest trees, respectively. However, the four-gene data set generated only three most parsimonious trees, each of 10910 steps (CI = 0.36, RI = 0.43). The only differences among the three most parsimonious trees involved the relationships among five genera of Ranunculaceae and among three genera of Papaveraceae (Fig. 4). Bootstrap support was generally higher with four genes than with individual genes (Table 4), in agreement with other comparisons involving the combination of data sets (e.g., Savolainen et al., 2000; Soltis et al., 2000).

In the MP analysis of the four-gene data set, a well-supported (99%) Ranunculales are sister to all other eudicots. Following Ranunculales, the shortest trees placed a weakly supported (65%) Proteales clade as sister to the rest of the eudicots (59%). Within Proteales, Nelumbonaceae are sister to a clade (67%

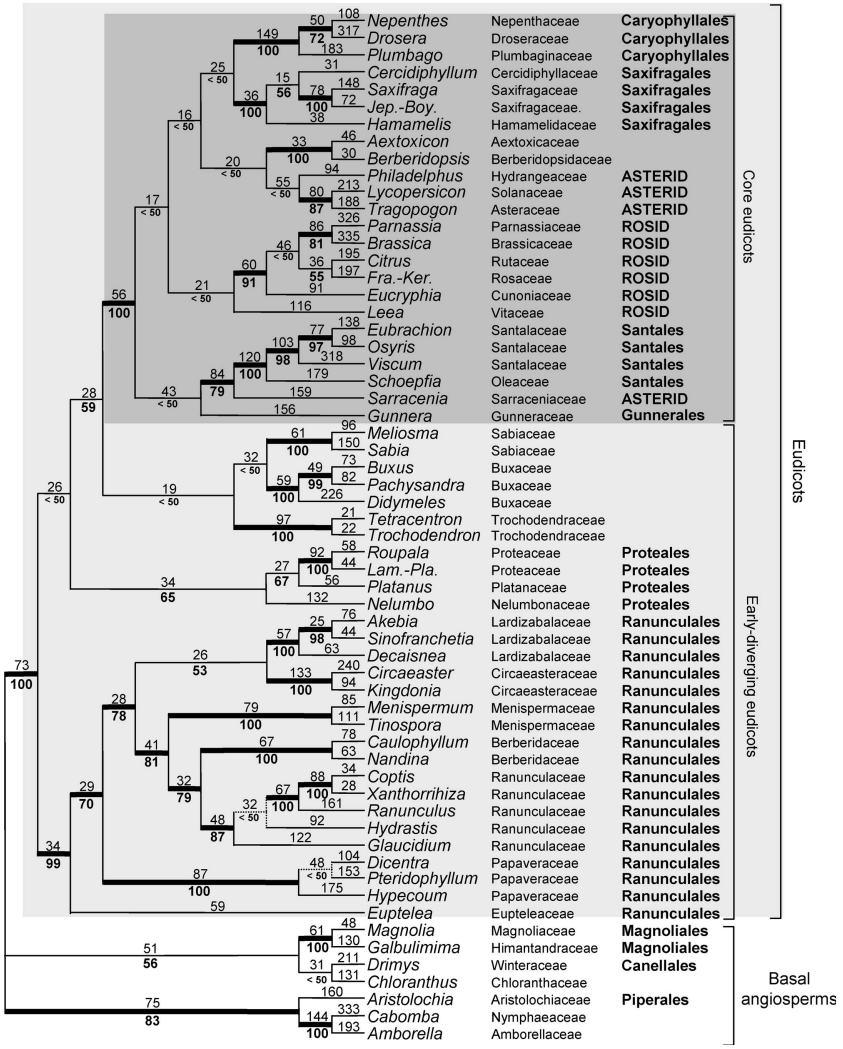

Fig. 4. One of three most parsimonious trees based on the combination of *atpB*, *rbcL*, 18S rDNA, and 26S rDNA sequences. Numbers along branches, dotted lines, and bold lines are as in Fig. 2. Abbreviations for several genera are as follows: *Jep.*, *Jepsonia*; *Boy.*, *Boykinia*; *Fra.*, *Fragaria*; *Ker.*, *Kerria*; *Lam.*, *Lambertia*; *Pla.*, *Placospermun*.

Table 4
Comparison of bootstrap support for major groups sensu APG II classification (2002) in separate and combined analyses

Clades	*rbcL*	*atpB*	18S rDNA	26S rDNA	Chloroplast	Nuclear	Three gene	Four gene
Eudicots	99	88	—	88	100	95	100	100
Buxaceae	90	76	—	89	99	96	100	100
Sabiaceae	92	72	—	100	99	100	99	100
Trochodendraceae	100	99	94	100	100	77	100	100
Proteales	—	62	—	—	84	—	86	65
Proteaceae	—	98	—	—	93	—	88	67
Ranunculales	87	51	—	53	98	68	99	99
Berberidaceae	—	100	90	99	100	100	100	100
Circaeasteraceae	100	99	98	99	100	100	100	100
Lardizabalaceae	97	100	91	98	100	100	100	100
Menispermaceae	100	100	72	100	100	100	100	100
Papaveraceae	82	83	76	79	100	93	100	100
Ranunculaceae	90	—	—	—	80	55	91	87
Core eudicots	61	100	—	—	100	53	100	100
Ranunculaceae + Berberidaceae	—	82	—	—	92	—	89	79
Ranunculaceae + Berberidaceae + Menispermaceae	—	64	—	—	66	—	76	81
Eupteleaceae sister to other Ranunculales	—	—	—	54	—	70	—	70
Papaveraceae sister to other Ranunculales	70	—	—	—	70	—	—	
Ranunculales sister to other eudicots	—	58	—	—	80	—	70	—

Note. Dashes indicate bootstrap values <50%.

support) of Proteaceae + Platanaceae. The low support for the monophyly of Proteales (65%) appears to be the effect of 26S rDNA data, which place Platanaceae as sister to Nelumbonaceae + Proteaceae rather than Nelumbonaceae as sister to Proteaceae + Platanaceae. The monophyly of Proteales was supported by 84 and 86% bootstrap values in analyses of the chloroplast and *rbcL atpB*/18S rDNA partitions, respectively. Following Proteales, the relationships of the remaining early-diverging eudicots remain uncertain. In the strict consensus tree, Trochodendraceae are sister to a clade of Sabiaceae + Buxaceae s. l. (Buxaceae + Didymelaceae; see APG II, 2003), but none of these relationships receives support >50%.

Within Ranunculales, relationships are generally well resolved and strongly supported with four genes. Eupteleaceae, followed by Papaveraceae, appear as successive sisters to all other Ranunculales, with bootstrap support of 70 and 78%, respectively. In contrast, the earlier three-gene analyses (Hoot et al., 1999; Soltis et al., 2000), as well as our own three-gene study, placed Papaveraceae followed by Eupteleaceae as sister to other members of Ranunculales. However, support for Papaveraceae as sister to Eupteleaceae + the rest of Ranunculales with three genes was lower (Hoot et al., 1999: <50%; Soltis et al., 2000: 53%) than the placement here of Eupteleaceae as sister to Papaveraceae + rest of Ranunculales (70%).

Following Eupteleaceae and Papaveraceae, Lardizabalaceae + Circaeasteraceae are weakly supported sisters (53%) and are sister to a clade (81%) of Menispermaceae + (Berberidaceae + Ranunculaceae) (79%). These relationships are very similar to those revealed with three genes (Fig. 3B).

The ML tree from the four-gene analysis is very similar to those obtained with maximum parsimony (Fig. 5). Ranunculales are again sister to other eudicots and are followed successively by Proteales, Sabiaceae, Buxaceae, and Trochodendraceae. However, the branch lengths of these clades are very short, in agreement with the poor resolution and support obtained with parsimony.

The Bayesian analysis revealed the same topology as ML for early-diverging eudicots; relationships among core eudicots and among outgroups differed between the analyses, however (Fig. 6). Most of relationships among early-diverging eudicots received a posterior probability of 1.00, including the following: the sister group relationship of Ranunculales to all other eudicots, most of relationships among Ranunculales including basal-most position of *Euptelea* in the order, and relationships among Proteales, Trochodendraceae, Buxaceae, and Sabiaceae (Fig. 6).

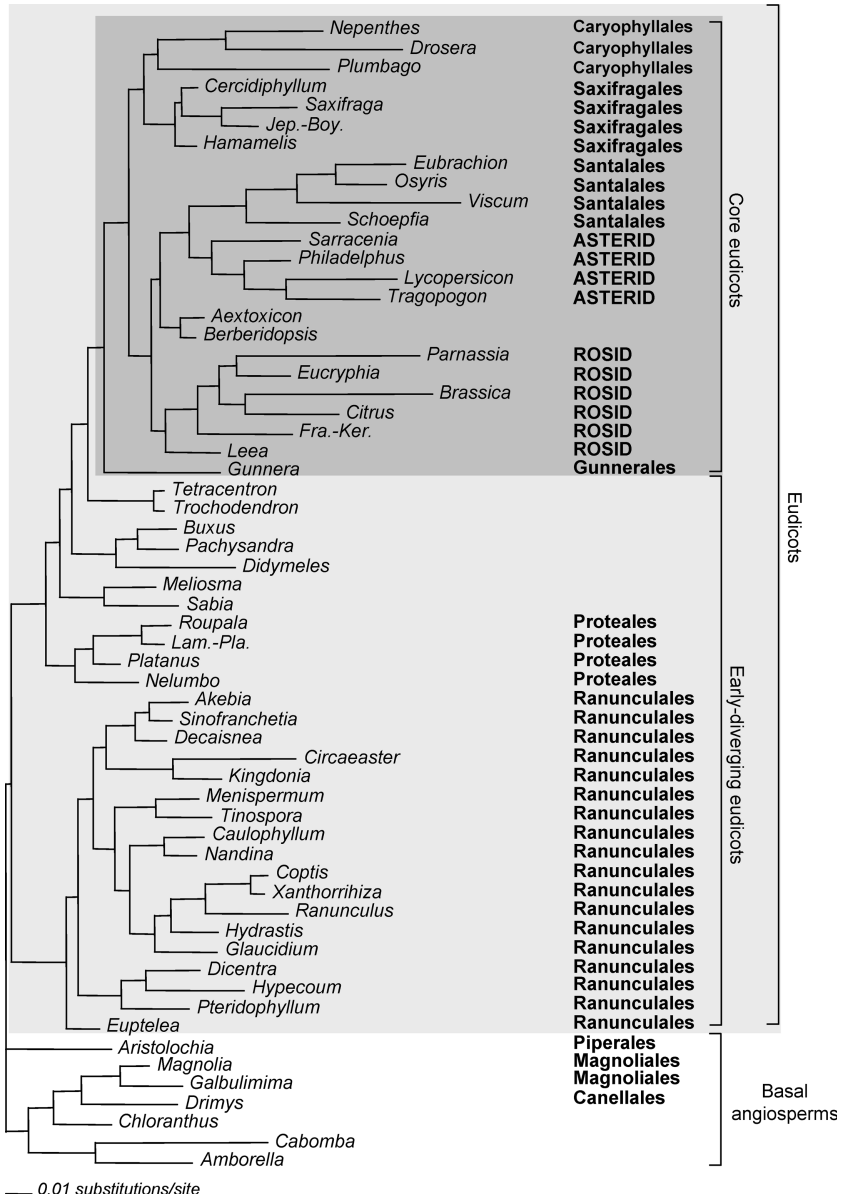

Fig. 5. Maximum likelihood tree generated from analysis of four-gene data set.

3.4. Character evolution

Most basal angiosperm lineages are woody, with the notable exceptions of Nymphaeaceae, some Piperales, and some Chloranthaceae (Cronquist, 1981; Doyle and Endress, 2000; reviewed in Zanis et al., 2003). In contrast, however, most Ranunculales are herbaceous. Ranunculales were long considered to be derived from Magnoliales and "primitively herbaceous" (Cronquist, 1968, 1988; Takhtajan, 1991, 1997). To evaluate the hypothesis of an ancestrally herbaceous habit for Ranunculales, we optimized habit, coded as woody vs. herbaceous.

Our reconstructions indicate that the ancestral state for several of major clades of eudicots is most likely woody. For example, the first-branching Saxifragales include *Daphniphyllum*, *Cercidiphyllum*, and Altingiaceae, all of which are woody; hence, the ancestral state reconstructed for this clade is clearly woody (Fishbein, in preparation). Similarly, Cornales followed by Ericales, both of which are woody, appear as sister to all other asterids (Albach et al., 2001; Bremer et al., 2002); the ancestral state for this large clade is reconstructed as woody. Dilleniaceae, which are sister to Caryophyllales, are woody as are the first-branching members of Caryophyllales, so this clade also appears to be ancestrally woody (Fig. 7). The ancestral state for the rosids is unclear. Within the rosids, Vitaceae (which are woody) are sister to all other rosids, but relationships among the

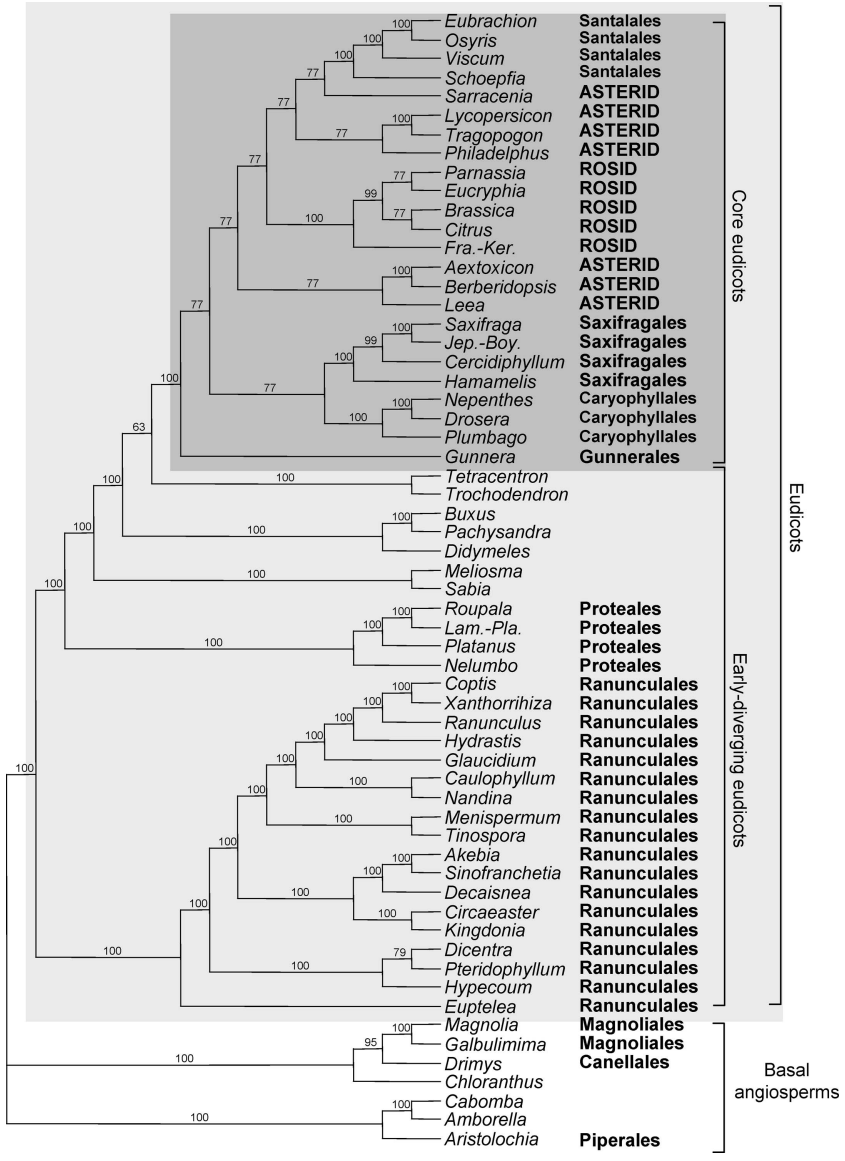

Fig. 6. Bayesian tree generated from analysis of four-gene data set. Posterior probability multiplied by 100.

remaining rosids are uncertain; thus, the rosids are scored as polymorphic.

Using the results of our four-gene analysis and the composite topologies we constructed for angiosperms (see Materials and methods), the woody habit is inferred to be ancestral not only for basal angiosperms, but also for several major clades of eudicots (Fig. 7). Furthermore, Ranunculales may not be ancestrally herbaceous, as long maintained. The ancestral condition for Ranunculales (and for the eudicots) depends on the relationships within Ranunculales and on the optimization assumptions that have been used. Using the three-gene topology (Hoot et al., 1999; Soltis et al., 2000), Papaveraceae are sister to the remainder of Ranunculales. With this topology, some character codings and the all most parsimonious states optimization, the ancestral state for Ranunculales is variously reconstructed as woody or ambiguous (Fig. 7). However, in our four-gene topology, Eupteleaceae are sister to all other Ranunculales (Figs. 4 and 5), and this placement has an important impact on character-state reconstruction for Ranunculales. With Eupteleaceae sister to all other Ranunculales, the ancestral condition for the order is always woody using all three optimizations (ACCTRAN, DELTRAN, and "all most parsimonious states"). Regardless of the topology for Ranunculales (and placement of Eupteleaceae and Papaveraceae), the ancestral state for the remaining eudicots is also the woody habit. The use of different topologies and character codings for various members of Ranunculales for core eudicots had no impact on this reconstruction.

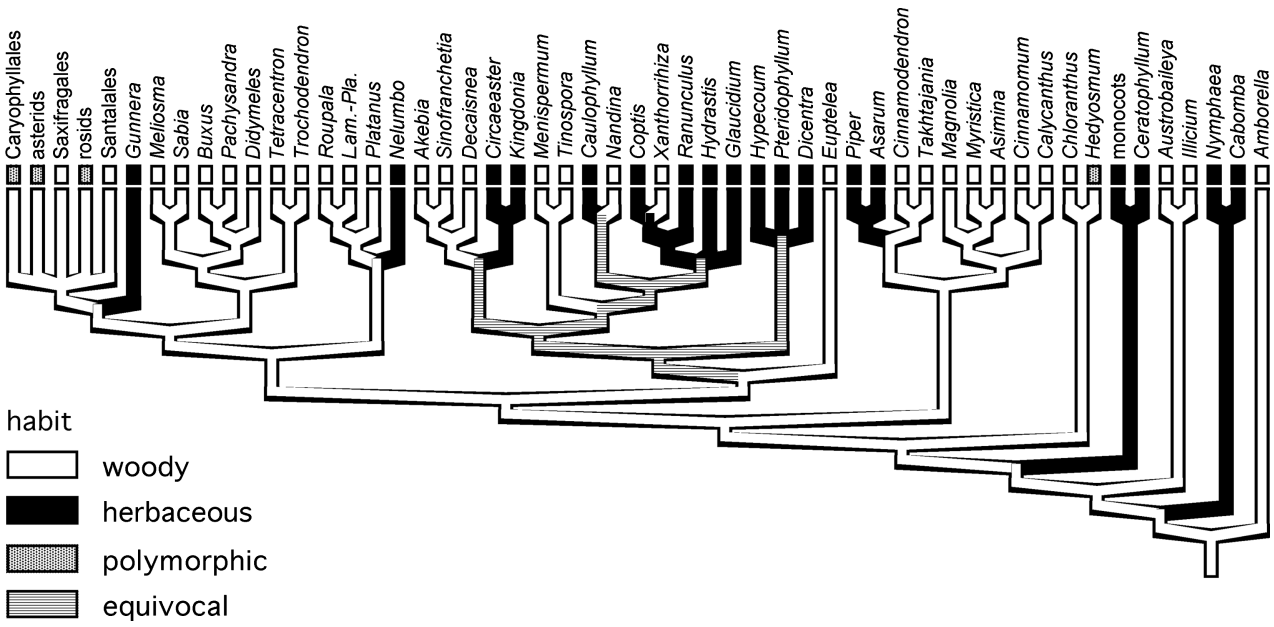

Fig. 7. MacClade reconstruction of the evolution of plant habit using the all most parsimonious states option. Lam.-Pla. = *Lambertia-Placospermum*.

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