Multigene Phylogeny of Land Plants with Special Reference to Bryophytes and the Earliest Land Plants

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A widely held view of land plant relationships places liverworts as the first branch of the land plant tree, whereas some molecular analyses and a cladistic study of morphological characters indicate that hornworts are the earliest land plants. To help resolve this conflict, we used parsimony and likelihood methods to analyze a 6,095-character data set composed of four genes (chloroplast *rbcL* and small-subunit rDNA from all three plant genomes) from all major land plant lineages. In all analyses, significant support was obtained for the monophyly of vascular plants, lycophytes, ferns (including *Psilotum* and *Equisetum*), seed plants, and angiosperms. Relationships among the three bryophyte lineages were unresolved in parsimony analyses in which all positions were included and weighted equally. However, in parsimony and likelihood analyses in which *rbcL* third-codon-position transitions were either excluded or downweighted (due to apparent saturation), hornworts were placed as sister to all other land plants, with mosses and liverworts jointly forming the second deepest lineage. Decay analyses and Kishino-Hasegawa tests of the third-position-excluded data set showed significant support for the hornwort-basal topology over several alternative topologies, including the commonly cited liverwort-basal topology. Among the four genes used, mitochondrial small-subunit rDNA showed the lowest homoplasy and alone recovered essentially the same topology as the multigene tree. This molecular phylogeny presents new opportunities to assess paleontological evidence and morphological innovations that occurred during the early evolution of terrestrial plants.

Introduction

Understanding the evolutionary history of bryophytes (mosses, liverworts, and hornworts) is compelling because they represent the first green plants to colonize the terrestrial environment, in the course of which they evolved numerous important adaptations, including alternation of gametophytic (haploid) and sporophytic (diploid) generations, elaborate gametophytes, specialized gametangia, and desiccation-resistant spore walls (Renzaglia et al. 2000). Spore microfossils of terrestrial (nonalgal) plants are known from the middle Ordovician (470 MYA) (Gray 1993); however, the earliest spore records identified as liverworts are from the lower Silurian (430 MYA) (Taylor 1995) and the earliest spore records for hornworts are from the upper Silurian (420 MYA) (Richardson 1985). Furthermore, unequivocal macrofossils of liverworts (*Pallavicinites*) and hornworts (*Notothylacites*) date to the upper Devonian (370 MYA) (Hueber 1961) and upper Cretaceous (70–90 MYA) (Nemejc and Pacltova 1974), respectively, i.e., long after their initial appearance. Determining which bryophyte group is the most ancient among extant land plants has proven difficult, and a myriad of phylogenetic hypotheses have been advanced with fundamentally different tree topologies. The long-standing concept of bryophyte monophyly was first challenged by morphological cladistic analyses which placed liverworts as the sister to all other land plants (Mishler and Churchill 1984). Analyses combining morphological characters with rRNA sequences (Mishler et al. 1994) and analyses of the chloroplast gene *rbcL* (Lewis, Mishler, and Vilgalys 1997) have generally supported a ''liverworts-basal'' topology (LBT) and placed either mosses or hornworts as sister to vascular plants (tracheophytes). More recently, the LBT was deemed the most parsimonious explanation to account for the presence and absence of three mitochondrial introns (Qiu et al. 1998).

Despite its wide acceptance, the LBT has been challenged by data derived from spermatozoid ultrastructure and spermatogenesis (Renzaglia and Duckett 1991) and, more recently (Garbary and Renzaglia 1998), by an extensive cladistic analysis of morphological and ultrastructural data that yielded a monophyletic moss-liverwort clade, with hornworts sister to all land plants. This ''hornworts-basal'' topology (HBT) was also obtained in analyses of nuclear small-subunit (nuSSU) rDNA (Hedderson, Chapman, and Rootes 1996), combined analysis of five chloroplast genes (Nishiyama and Kato 1999), and analyses of mitochondrial *cox3* (Malek et al. 1996), *nad5* (Beckert et al. 1999), and mitochondrial small-subunit (mtSSU) rDNA (Duff and Nickrent 1999). In an effort to distinguish between the LBT and the HBT and to provide an improved understanding of land plant phylogeny, we generated additional sequence data to allow the construction of a multigene data set representing all three plant genomes and the same suite of exemplar taxa.

Materials and Methods

Ingroup and Outgroup Selection

Sequences were obtained from chloroplast SSU (cpSSU) rDNA, chloroplast *rbcL*, nuSSU rDNA, and

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Abbreviations: HBT, hornwort basal topology; LBT, liverwort basal topology.

Key words: land plant phylogeny, embryophyte, hornwort, liverwort, moss, *rbcL*, small-subunit ribosomal DNA.

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Table 1
GenBank Accession Numbers for Taxa Used in Multigene Analysis of Land Plant Phylogeny

	rbcL	cpSSU rDNA	nuSSU rDNA	mtSSU rDNA
Glycine max	Z95552	X06428	X02623	M16589
Nicotiana tabacum	Z00044	Z00044	**AF161095**	U82638
Zea mays	X86563	X86563	U42796	X00894
Magnolia × *soulangeana*/*M.* × *soulangeana*/*Magnolia acuminata*/*Magnolia grandiflorum*	M58393	**AF244557**	D29776	**AF161089**
Nymphaea odorata/*Nymphaea tuberosa*/*Nymphaea tuberosa*/*Nymphaea* sp.	M77034	**AF244560**	L24404	**AF161091**
Gnetum gnemon/*Gnetum leyboldii*/*Gnetum gnemon*/*Gnetum montanum*	L12680	**AF244555**	U42416	**AB029358**
Ephedra tweediana/*Ephedra trifurca*/*Ephedra torreyana*/*Ephedra antisyphitica*	L12677	U24584	U42414	**AF244552**
Juniperus conferta/*Juniperus virginiana*/*Juniperus chinensis*/*Juniperus virginiana*	L12573	U24586	D38243	**AF058658**
Pinus thunbergii/*Pinus thunbergi*/*Pinus elliotii*/*Pinus strobus*	D17510	D17510	D38245	AF058659
Cycas circinalis/*Cycas revoluta*/*Cycas taitungensis*/*Cycas revoluta*	L12674	**AF244551**	D85297	**AB029356**
Ginkgo biloba	D10733	**AF244554**	D16448	AB029355
Diphasiastrum digittatum/*Diphasiastrum digittatum*/*Diphasiastrum tristachyum*/*Diphasiastrum digittatum*	L11055	U24587	U18511	AF058667
Huperzia selago/*Huperzia lucidula*/*Huperzia lucidula*/*Huperzia lucidula*	Y07934	**AF244556**	U18505	AF058668
Selaginella deflexa/*Selaginella apoda*/*Selaginella vogelii*/none	AF093253	U24591	X75517	**None**
Isoëtes melanopoda/*Isoëtes melanopoda*/*Isoëtes durieui*/*Isoëtes hystrix*	L11054	U24585	X83521	AF058669
Equisetum arvense/*Equisetum arvense*/*Equisetum hyemale*/*Equisetum hyemale*	L11053	U24593	X78890	AF058663
Botrychium biternatum/*Botrychium biternatum*/*Botrychium lunaria*/*Botrychium dissectum*	L13474	U24581	U18623	AF058661
Ophioglossum engelmannii/*Ophioglossum engelmannii*/*Ophioglossum petiolatum*/*Ophioglossum vulgare*	L11058	U24589	U18515	AF058664
Adiantum raddianum/*Adiantum pedatum*/*Adiantum raddianum*/*Adiantum raddianum*	U05906	**AF244549**	U18621	AF058660
Angiopteris evecta/*Angiopteris evecta*/*Angiopteris lygodiifolia*/*Angiopteris lygodiifolia*	L11052	U24580	D85301	**AB029352**
Psilotum nudum	L11059	U24590	U18519	**AF244566**
Sphagnum palustre	L13485	U24592	Y11370	AF058670
Takakia lepidozioides	**AF244565**	AF058678	U18526	AF058672
Polytrichum commune	U87087	**AF244563**	U18518	**AF244564**
Marchantia polymorpha	X04465	X04465	X75521	M68929
Calypogeia muelleriana/*Calypogeia muelleriana*/*Calypogeia arguta*/*Calypogeia muelleriana*	U87065	**AF244550**	X78439	AF058674
Megaceros enigmaticus/*Megaceros enigmaticus*/*Megaceros tosanus*/*Megaceros tosanus*	L13481	**AF244558**	**AF244559**	AF058675
Phaeoceros laevis	**AF244562**	**AF244561**	U18491	AF058677
Chara connivens/*Chara* sp. 1/*Chara connivens*/*Chara* sp. 2	L13476	X75519	U18493	**AF191800**
Spirogyra maxima/*Spirogyra maxima*/*Spirogyra grevilleana*/*Spirogyra grevilleana*	L11057	U24596	U18523	**AF191801**

NOTE.—Multiple placeholder species for a single terminal are indicated by a slash and the order listed follows the gene order. GenBank accession numbers in bold are new reports generated for this study.

mtSSU rDNA for representatives of all major extant land plant lineages. Sampling included two hornworts (*Phaeoceros* and *Megaceros*), two liverworts (*Marchantia* and *Calypogeia*), three mosses (*Takakia, Polytrichum*, and *Sphagnum*), four lycophytes (*Selaginella, Isoëtes, Huperzia,* and *Diphasiastrum*), six ferns (*Psilotum, Ophioglossum, Botrychium, Angiopteris, Equisetum, and Adiantum*), six gymnosperms (*Cycas, Ginkgo, Juniperus, Pinus, Gnetum,* and *Ephedra*), and five angiosperms (*Nymphaea, Magnolia, Zea, Nicotiana,* and *Glycine*). *Chara* and *Spirogyra* (green algae) were used as outgroups. Taxa were initially selected based on the presence of one or more of the four gene sequences in GenBank, as well as a desire to represent a wide range of taxonomic diversity. From this list, sequences from 30 genera were obtained from GenBank or generated for this study. For 10 of the 28 ingroup taxa, the same species was used for all four gene sequences (table 1). Placeholder species were used for the remaining 18 taxa: 8 cases with two species, 11 cases with three species, and for *Ephedra,* four different species were used. Of the 119 DNA sequences assembled, 27 were newly generated. As previously reported (Duff and Nickrent 1999), PCR amplifications of mtSSU rDNA from *Coleochaete* and *Selaginella* failed; hence, *Coleochaete* was not used as an outgroup, and *Selaginella* represent-

ed the only case of a missing ingroup sequence in this study. Complete voucher information for newly generated sequences, full scientific names, and GenBank numbers for sequences of all taxa are given in table 1.

Molecular Methods

The protocols used here for extracting genomic DNA, PCR, cloning, and sequencing have previously been reported (Nickrent 1994; Qiu et al. 1998; Duff and Nickrent 1999; Chaw et al. 2000). The primers used to amplify and sequence the newly generated sequences were cited in the following: *rbcL* (Soltis and Soltis 1998), cpSSU rDNA (Nickrent, Duff, and Konings 1997), nuSSU rDNA (Nickrent and Starr 1994), and mtSSU rDNA (Duff and Nickrent 1999).

Alignments

Separate gene and multigene alignments were conducted manually using SeqApp (Gilbert 1993). Alignment of *rbcL* and cpSSU rDNA sequences was trivial, given essentially no length variation, whereas for nuclear and mitochondrial SSU rDNA sequences, alignments were guided by reference to published higher-order structures (Nickrent and Soltis 1995; Duff and Nickrent 1999). Terminal priming regions were excluded from all sequences, as were regions where alignment was ambiguous or where extensive length variation occurred (e.g., introns in mtSSU rDNA; see Duff and Nickrent 1999). There were (with reference to Glycine sequences) 51 such sites for *rbcL*, 63 for cpSSU rDNA, 133 for nuSSU rDNA, and 633 for mtSSU rDNA, for a total of 909 sites excluded from the multigene data set. The key to excluded regions and the final alignment (6,095 positions: *rbcL* = 1,351, cpSSU rDNA = 1,480, nuSSU rDNA = 1,720, and mtSSU rDNA = 1,544) can be found at http://www.science.siu.edu/landplants/Alignments/Alignments.html.

Parsimony and Likelihood Analyses

There currently exists conflicting information in the literature as to whether *rbcL* third codon positions are saturated, or, more specifically, whether the process of substitution has become so randomized as to preclude the use of this position for ascertaining deep phylogenetic relationships. Evidence that third-codon-position transitions are effectively saturated has been presented by Goremykin et al. (1996) and Chaw et al. (2000). Conversely, Lewis, Mishler, and Vilgalys (1997) showed that trees generated from only third positions are nearly identical to trees produced from all sites and that these trees agree with independent evidence. Hence, they concluded that, despite being highly variable, third positions retain phylogenetic signal across all land plants. A similar conclusion was reached by Källersjö et al. (1998). To test for saturation, we plotted uncorrected p-distances from first-, second-, and third-position transitions and transversions against corrected F84 distances (Felsenstein 1984).

Four versions of the multigene data set were constructed and analyzed with either maximum parsimony (MP) or maximum likelihood (ML). In the first version, all positions, including *rbcL* third codon positions, were included and were weighted equally. Analyses of this version were called MP+3Ti and ML−rates. The second version also included all positions, but differential weighting (ranging from 0.5 to 1.0) was applied to third codon positions. Analyses of this version were dubbed MP+weights. The third version excluded *rbcL* third-codon-position transitions, which was accomplished by recoding all third-position nucleotides as either R (A or G) or Y (C or T). Analyses of this version were called MP−3Ti and ML−3Ti. In the fourth version, all positions were included, and site-to-site rate variation was accounted for using the program DNArates (S. Pract, R. Overbeek, and G. Olsen, personal communication; available at the Ribosomal Database Project website). Analysis of this version was called ML+rates.

All MP analyses were conducted using PAUP* (Swofford 1998). For these analyses, the separate and combined gene matrices were analyzed either with all characters equally weighted or with unequal weighting (see above). Heuristic search strategies with random taxon addition (100 replicates), MULPARS on, and tree bisection-reconnection (TBR) branch swapping was employed. MP bootstrap (BS) support was assessed using PAUP*, employing a heuristic search strategy with 100 replications (1,000 for the multigene data set).

ML analyses employed fastDNAml, version 1.06, rewritten to run in parallel, on 8–16 nodes of the STARRS/SP cluster (IBM RS/6000 computer platform, located at Indiana University). The F84 model (Felsenstein 1984) as implemented in PHYLIP (Felsenstein 1993) was used, with the initial transition/transversion (ti/tv) ratio estimated using PUZZLE, version 4.02, under the Tamura-Nei model of evolution with parameter estimation set to ''approximate'' (Strimmer and von Haeseler 1996). Ten initial ML trees were inferred for each individual and the combined data set by randomizing ''input'' order with jumble and using ''global'' swapping across all nodes (equivalent to subtree-pruning-regrafting of PAUP*). The optimal tree (best log likelihood score) was then input into PAUP* to reoptimize the ti/tv ratio using a model that incorporated variability in rates of change. We used the F84 evolutionary model assuming a discrete gamma distribution with four categories of site-to-site rate variability. The resulting ti/tv ratio was used to infer a new tree as above, further optimizing branch lengths. This tree and the optimized ti/tv ratio were then used to estimate evolutionary rates of change for each sequence position by partitioning the sites into 35 ''rate'' categories using DNArates. A new ML tree, incorporating the rate categories and the reoptimized ti/tv ratio, was then inferred. This new optimal tree was then used for a second round of rate estimation and tree inference. This process was iterated until a stable topology was achieved.

ML bootstrapping with and without rates used SEQBOOT (PHYLIP) to generate 100 pseudoreplicate data sets that were analyzed using fastDNAml, and the re-

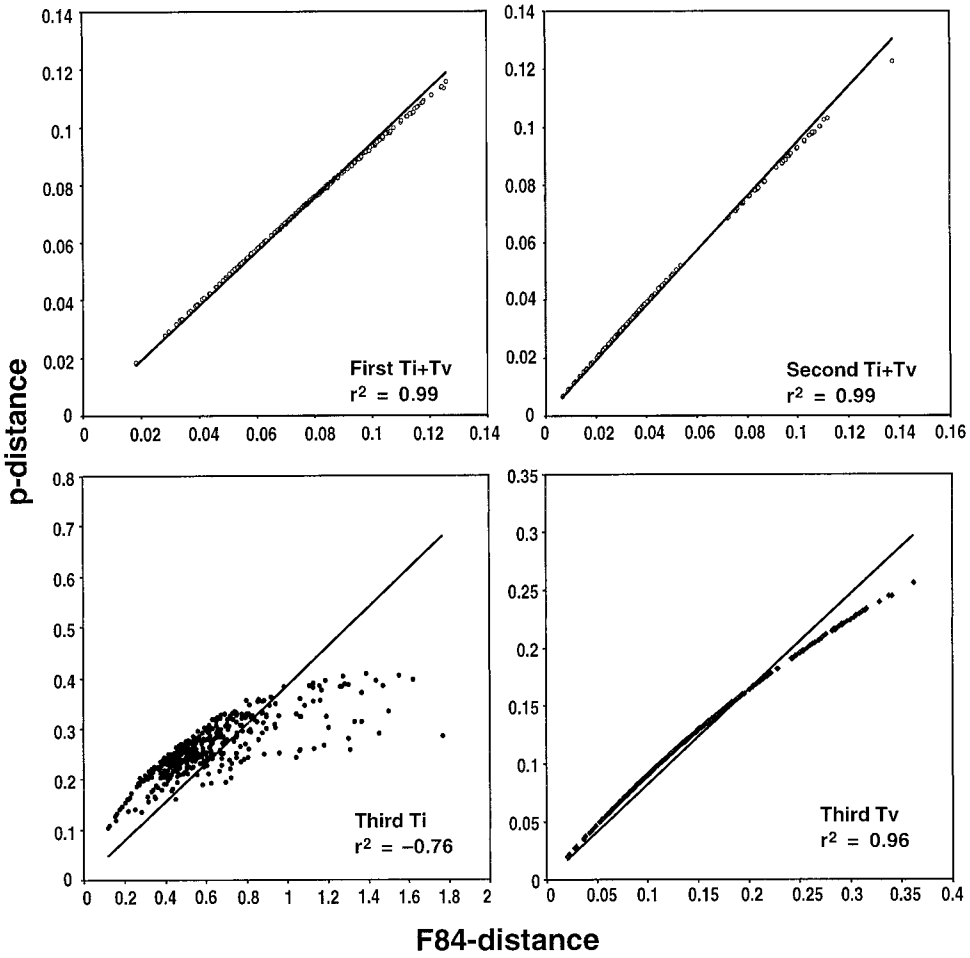

FIG. 1.—Plots of uncorrected pairwise sequence divergence (p-distances) versus corrected distances (F84-distance) for first, second, and third codon positions in *rbcL*. Transitional (ti) and transversional (tv) substitutions are combined for first and second positions, whereas they are plotted separately for third positions. Deviation from the diagonal trend line, as is evident for third-codon-position transitions, indicates saturation. A total of 427 pairwise comparisons are represented in each panel. Eight ingroup-to-algal-outgroup comparisons for third-position transitions were excluded because the observed dissimilarity exceeded 0.75.

sulting bootstrap numbers were generated using CONSENSE (PHYLIP). The individual trees from the 100 pseudoreplicates were analyzed as above using fastDNAml, randomizing the input order, and allowing swapping across all nodes. Each initial ''no-rates'' topology was then used to infer rate categories incorporating the optimized ti/tv ratio. A second round of rate category generation and tree inference was performed, and the final 100 trees were imported into CONSENSE to generate bootstrap node values.

Alternative topologies, evaluated under ML and MP, were determined using the parametric K-H test (Kishino and Hasegawa 1989) and the nonparametric Templeton (1983) ranked-sums test as implemented in PAUP*. Decay analyses (Bremer 1994) were performed with MP using PAUP* and AUTODECAY (Eriksson 1998).

Results

Composition Bias and Saturation

Nucleotide compositions for the entire multigene data set varied little among taxa (A = 0.253, C = 0.228, G = 0.292, T = 0.227), with an average A+T composition of 48%. A chi-square test of homogeneity of base frequencies across taxa gave 45.607 (df = 87, P = 0.9999); hence, nucleotide composition was homogeneous. Homogeneity was also seen for all of the separate gene partitions with the exception of *rbcL*. Chi-square tests of homogeneity showed that *rbcL* first and second codon positions were homogeneous, whereas third positions (with A+T averaging 72% and ranging from 46% in *Selaginella* to 85% in *Marchantia*) were significantly heterogeneous across taxa. These results agree with those reported for *rbcL* by Lewis, Mishler, and Vilgalys (1997) and Nishiyama and Kato (1999) and therefore require the use of sequence evolution models that do not assume compositional equality. In tests for saturation using distance plots (fig. 1), first- and second-position transitions and transversions and third-position transversions were significantly linear, whereas third-position transitions were significantly nonlinear. This result parallels those of Goremykin et al. (1996) and Chaw et al. (2000) and, we believe, indicates third-position transitional saturation for *rbcL*.

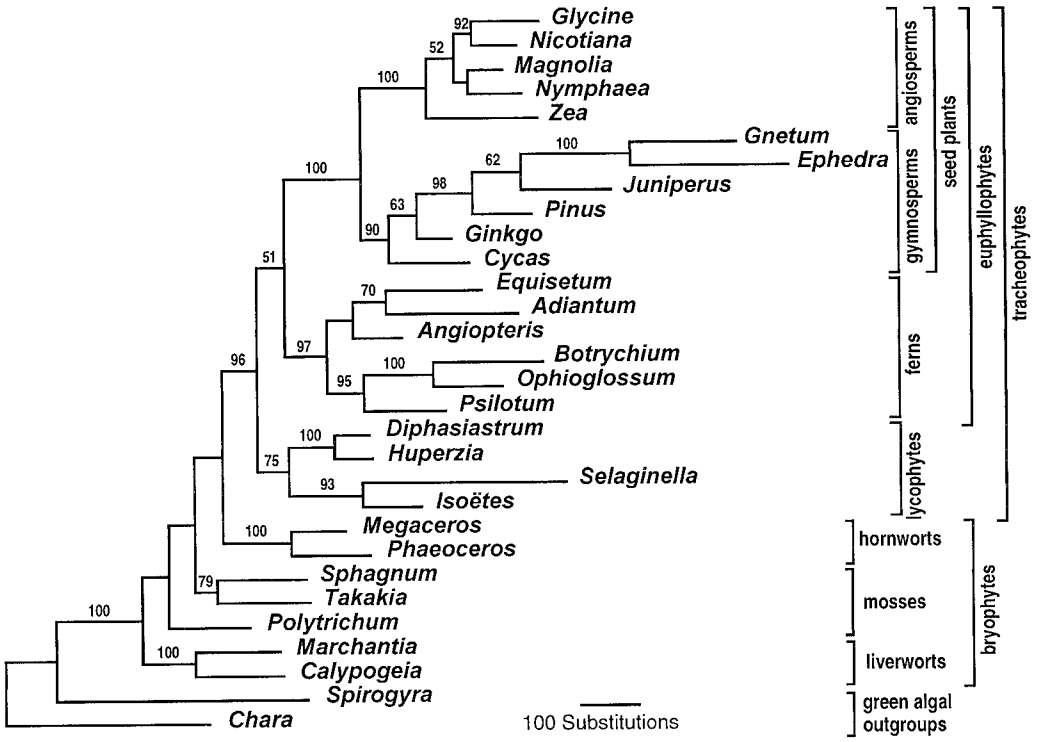

FIG. 2.—Land plant relationships derived from an MP+3Ti analysis of the four-gene data set. A single tree (length = 7,074) was obtained with a consistency index (excluding uninformative sites) of 0.3900, a retention index of 0.4756, and a rescaled consistency index of 0.2332. Values above the branches are bootstrap percentages derived from 100 replications.

Phylogenetic Relationships Recovered Using All Analytical Methods

Of the 6,095 characters in the alignment, 1,037 (17%) were variable but parsimony-uninformative, and 1,219 (20%) were parsimony-informative. For the multigene data set, MP+3Ti analysis resulted in one tree of length 7,074 (fig. 2). MP−3Ti analysis yielded a single tree of length 5,252 (fig. 3). These two trees are highly congruent for relationships among tracheophytes but differed with regard to bryophyte relationships (see below).

As shown in figures 2 and 3 and table 2, MP+3Ti, MP−3Ti, ML−rates, and ML+rates analyses all yielded high BS values for the majority of land plant clades, including angiosperms, gymnosperms, seed plants, ferns (including *Psilotum* and *Equisetum*), lycophytes, tracheophytes, liverworts, and hornworts. When BS support >70% is considered significant (Hillis and Bull 1993), the following percentages of total nodes were supported as significant among the various analyses: MP+3Ti (70%), MP−3Ti (78%), ML−rates (74%), and ML+rates (81%). The MP and ML analyses gave strong support for a clade composed of all vascular plants (tracheophytes), although this was not the case among the nonmitochondrial genes analyzed individually. Moderate (MP) to high (ML) BS values were obtained for lycophyte monophyly. All methods gave high BS support for lycophytes as the sister to all other vascular plants, consistent with other molecular (Raubeson and Jansen 1992) and morphological (Kenrick and Crane 1997) analyses. The two heterosporous genera (*Selaginella* and *Isoëtes*) are sisters, as are the two homosporous genera (*Huperzia* and *Diphasiastrum*); these relationships generally follow current classifications (Kenrick and Crane 1997). The monophyly of ferns, here including the horsetail *Equisetum* and the whiskfern *Psilotum*, has high BS and decay analysis support. Within this clade, *Psilotum* is strongly supported as sister to the eusporangiate ferns *Botrychium* and *Ophioglossum*, a relationship now well documented (Manhart 1994; Pryer, Smith, and Skog 1995; Wolf 1997). Weak support exists for the placement of *Angiopteris* as sister to *Equisetum* and *Adiantum*, although this topology was also obtained in a combined analysis of *rbcL* and morphological characters (Pryer, Smith, and Skog 1995). Seed plants are clearly resolved as monophyletic, with moderate (MP) to strong (ML) support for a clade of extant gymnosperms. A clade of gnetophytes (*Gnetum* and *Ephedra*) and conifers (*Pinus* and *Juniperus*) is very strongly supported (98%–100% BS, decay value of 24 steps), in agreement with two recent multigene studies of seed plant phylogeny (Bowe, Coat, and dePamphilis 2000; Chaw et al. 2000). Angiosperms are strongly supported as monophyletic in all analyses and with all separate gene partitions (table 2).

Phylogenetic Relationships that Differed Among the Various Analyses

Several differences can be seen when the MP+3Ti tree (fig. 2) and the MP−3Ti tree (fig. 3) are compared. Euphyllophytes (vascular plants exclusive of lycophytes; Kenrick and Crane 1997) are strongly supported using ML+rates and MP−3Ti. Different relationships

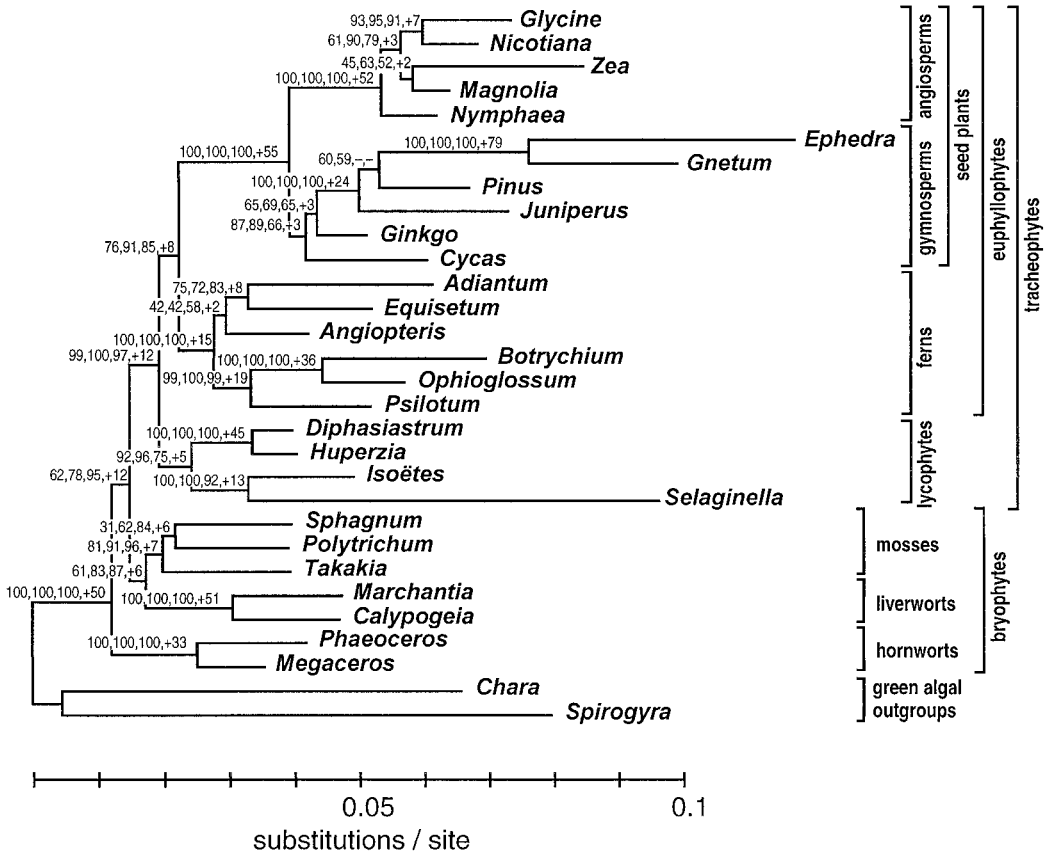

FIG. 3.—Land plant relationships derived from an MP−3Ti analysis of the four-gene data set. A single tree (length = 5,251) was obtained with a consistency index (excluding uninformative sites) of 0.4312, a retention index of 0.5275, and a rescaled consistency index of 0.2941. Branch lengths shown are derived from the ML+rates analysis (log likelihood = −37,923.99574, ti/tv = 2.25). Numbers at nodes (left to right): ML−rates BS values, ML+rates BS values, MP−3Ti BS values, and decay index. Nodes not present are indicated by dashes.

Table 2
Bootstrap[a] Percentages for Selected Land Plant Clades

	GENE						
CLADE	*rbcL*[b] (35 trees, HI = 0.679)	cpSSU (30 trees, HI = 0.524)	nuSSU (2 trees, HI = 0.578)	mtSSU (2 trees, HI = 0.435)	No mtSSU (1 tree, HI = 0.611)	No *rbcL* (1 tree, HI = 0.524)	Multigene (1 tree, HI = 0.569)
Angiosperms	98/99/86	85/84/81	100/100/100	100/100/100	100	100/100/100	100/100/100
Gymnosperms	–/–60	–/–/–	–/22/33	51/71/–	78	–/60/51	87/89/66
Seed plants	99/100/99	99/100/92	99/91/100	99/99/100	100	100/100/100	100/100/100
Ferns[c]	40/63/47	–/–/–	30/–/–	100/100/100	80	100/100/99	100/100/100
Psilotum sister to eusporangiates[d]	70/92/50	53/–/65	59/65/52	74/73/75	97	100/100/100	99/100/99
Euphyllophytes[e]	–/–/–	–/–/–	45/58/28	98/98/87	55	94/96/88	76/91/85
Lycophytes	76/80/–	–/–/–	–/–/36	98/99/93	—	82/82/62	92/96/75
Tracheophytes	25/45/47	–/–/–	–/–/–	97/96/97	53	100/99/96	99/100/97
Liverworts	41/70/60	100/100/100	–/–/–	100/100/100	80	100/100/100	100/100/100
Mosses	–/–/–	–/–/–	–/–/–	92/93/90	—	97/99/90	81/91/96
Mosses sister to liverworts	–/–/–	–/–/23	–/–/–	78/78/78	—	94/94/84	61/83/87
Hornworts	98/96/77	100/100/100	86/83/96	100/100/100	100	100/100/100	100/100/100
Hornworts basal	–/–/–	48/37/57	–/–/–	74/56/74	—	97/96/96	62/78/95

NOTE.—HI = homoplasy index minus uninformative sites.

[a] Maximum likelihood (ML) without DNArates/ML with DNArates/maximum parsimony (MP); MP only for no mtSSU. 100 replications for all ML and MP analyses except MP Multigene, which used 1,000 replications. A dash indicates that a clade not present in ML or strict-consensus MP trees.

[b] Outgroups (*Chara* and *Spirogyra*) not monophyletic; third-position transversions only for MP.

[c] Including *Equisetum* and *Psilotum.*

[d] Here, the ferns *Botrychium* and *Ophioglossum.*

[e] Vascular plants exclusive of lycophytes.

among the five angiosperm taxa were obtained following analyses of the multigene data using MP and ML. For MP+3Ti analyses, *Zea* was sister to the rest, albeit with low BS support (fig. 2). The MP−3Ti, MP+weights, ML−rates, and ML+rates analyses all resolved *Nymphaea* as basalmost with higher BS support, a result consistent with recent global angiosperm analyses (Parkinson, Adams, and Palmer 1999; Qiu et al. 1999; P. S. Soltis, D. E. Soltis, and Chase 1999; Bowe, Coat, and dePamphilis 2000; Chaw et al. 2000; D. E. Soltis, P. S. Soltis, and Chase 2000). Both the MP+3Ti and the MP−3Ti analyses placed *Juniperus* as sister to the gnetophytes, although in both cases BS support for this relationship was low. In contrast, ML both with and without rates placed *Pinus* as sister to the gnetophytes, with *Juniperus* sister to that entire clade.

The most striking differences between the MP+3Ti and MP−3Ti analyses involved bryophyte relationships. The single shortest tree recovered from the multigene data set using MP+3Ti (fig. 2) showed the liverworts *Marchantia* and *Calypogeia* as sister to all other land plants (the LBT), with paraphyletic mosses and the hornworts *Megaceros* and *Phaeoceros* sister to the tracheophytes. None of these bryophyte relationships received BS support >50%. Mosses were not supported as monophyletic using any of the nonmitochondrial partitions separately (table 2), but only achieved monophyly in the multigene data sets in which *rbcL* third position transitions were excluded or downweighted (MP+weights). A similar result was obtained for the sister group relationship between mosses and liverworts. One topological difference occurred within the moss clade when using ML−rates as opposed to ML+rates and MP−3Ti. The former method placed *Polytrichum* as sister to a *Takakia/Sphagnum* clade (tree not shown), whereas the latter methods placed *Takakia* as sister to *Sphagnum/Polytrichum* (fig. 3). For the MP+weights analysis, the HBT was obtained using all weights up to 0.93, whereas the LBT was retrieved with weights above this threshold.

In general, BS support for most clades was equivalent or higher for ML+rates than for ML−rates; this was true for the multigene data set and, to a lesser extent, for analyses of individual genes (table 2). Such an increase in BS support can be seen for the HBT, where the ML+rates and ML−rates BS values were 62% and 78%, respectively. Three clades in the multigene analysis showed marked differences in BS values between ML+rates and MP: gymnosperms, lycophytes, and HBT (table 2). For the HBT, the lower BS values obtained when using ML−rates (62%) or ML+rates (78%) as compared with MP (95%) may be attributed to the influence of the *rbcL* partition. Of the four partitions, only mtSSU and cpSSU rDNA analyzed separately retrieved the HBT (but with low to moderate BS support). Using ML and MP methods, combining the three SSU rDNA partitions gave BS values of 96%–97% for the HBT. That combined analyses can result in increased resolution and BS support for particular clades that were not well supported following individual analyses has been documented (Kluge 1989; Mishler et al. 1994; Soltis et al. 1998; Soltis, Soltis, and Chase 1999). Moreover, clades that were not present following analyses of individual genes can appear and gain significant BS support upon combined analyses. Examples here include the euphyllophyte, fern, and gymnosperm clades using *rbcL*, cpSSU, and nuSSU rDNA separately and in combination.

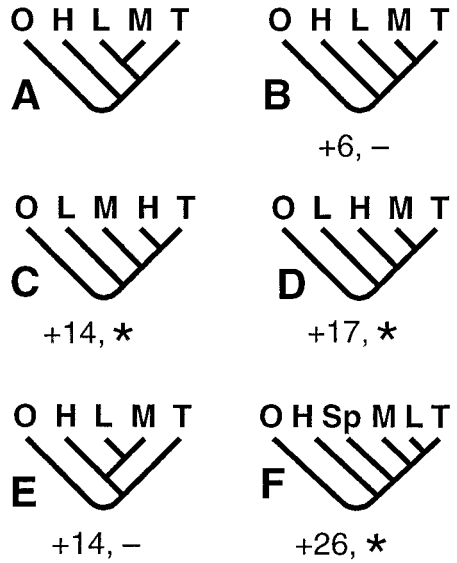

FIG. 4.—Alternative topologies imposed on the multigene data set: *A* (Hedderson, Chapman, and Rootes 1996; Garbary and Renzaglia 1998; Duff and Nickrent 1999; Nishiyama and Kato 1999; present work), *B* (Mishler et al. 1992), *C* (Lewis, Mishler, and Vilgalys 1997), *D* (Mishler and Churchill 1984), *E* (Garbary, Renzaglia, and Duckett 1993), and *F* (Malek et al. 1996). Numbers below trees represent additional steps compared with the shortest MP tree (length = 5,251). Results from K-H ML tests: * = statistically significantly different from best tree (*A*) at $P < 0.05$; — = not statistically significantly different. K-H MP and Templeton tests resulted in all alternate topologies being significantly different from tree *A* at $P < 0.05$. O = outgroup; H = hornworts; L = liverworts; M = mosses; Sp = Sphagnum; T = tracheophytes.

Decay Analyses and Hypothesis Testing

Decay analysis showed that 12 additional steps were required to collapse the hornworts-basal node (fig. 3). Five alternative topologies were tested with the multigene data set using the K-H test with ML and MP (fig. 4*A–F*). Two frequently cited LBTs depict either hornworts (fig. 4*C*) or mosses (fig. 4*D*) as sister to tracheophytes, but both were rejected as statistically worse ($P < 0.05$ level) than the ML tree. Similarly, the paraphyletic moss topology (fig. 4*F*) was significantly worse than the MP and ML trees. An alternative HBT (fig. 4*B*), in which liverworts and mosses were not sister taxa, added six steps to the most-parsimonious MP tree and could not be rejected relative to the ML tree. Finally, a monophyletic-bryophytes topology, although 14 steps longer than the MP tree, also could not be rejected relative to the ML tree. The K-H and Templeton tests conducted using MP as the criterion showed that all alternative trees (*B–F*) were significantly worse than tree *A* ($P < 0.05$).

Discussion

Outgroup Selection and Comparison Among the SSU Gene Partitions

This study is the first to sample among all major land plant lineages and across four genes representing the three plant subcellular genomes. Although sampling among tracheophytes was adequate, it is clear that additional bryophyte sequences are needed to place in context the results reported here. With the mtSSU rDNA data partition, the choice of outgroup is critical. In a previous analysis that utilized mtSSU rDNA (Duff and Nickrent 1999), basal land plant relationships were not unambiguously resolved with MP (although the HBT was retrieved with ML). This result was likely a consequence of using an algal outgroup (*Prototheca*) whose mtSSU sequence was much more distantly related to land plant sequences than the charophycean algal sequences used here. Pairwise comparisons of Kimura two-parameter distances using mtSSU rDNA gave the following results: *Chara* to *Phaeoceros* = 0.114, *Chara* to *Glycine* = 0.134, *Glycine* to *Phaeoceros* = 0.110, *Prototheca* to *Phaeoceros* = 0.342, *Prototheca* to *Glycine* = 0.364, and *Prototheca* to *Chara* = 0.347. These distances show that *Chara* is patristically closer to land plants than *Prototheca* (and hence probably a more appropriate outgroup), and branch lengths from *Chara* to ingroup taxa were not disproportionately long so as to perturb phylogenetic relationships (Felsenstein 1978).

Lower homoplasy in mitochondrial genes may derive from generally lower mutation rates as compared with nuclear or chloroplast genes (Wolfe, Li, and Sharp 1987; Palmer 1990; Wolfe 1996) and further highlights their utility in addressing ancient land plant relationships. Chloroplast SSU rDNA had the second lowest amount of homoplasy. Although this gene alone contains insufficient phylogenetic signal to resolve many clades, it did recover the liverwort clade and the HBT, two relationships that were not obtained using the longer and more variable nuSSU rDNA alone (table 2). The poor overall performance of nuSSU rDNA in resolving land plant relationships cannot be attributed to insufficient taxon sampling in this study, as similarly poor results were obtained in a recent study that utilized 93 ingroup sequences (Soltis et al. 1999).

Influence of the *rbcL* Partition on Resulting Trees

Chloroplast *rbcL* alone recovered a number of land plant clades but contained the highest level of homoplasy among the four gene partitions (table 2). When *rbcL* was analyzed alone using MP with all positions equally weighted, three trees were obtained that supported the LBT (62% BS support; results not shown). Removal of all third positions resulted in extensive loss of resolution across the entire tree, in agreement with Lewis, Mishler, and Vilgalys (1997) and Källersjö et al. (1998). Removal of third-position transitions resulted in a tree with no BS support for any of the major land plant clades. These exercises demonstrated that most of the phylogenetic signal in *rbcL* resides in third codon positions, specifically, third-position transitions. When the *rbcL* partition was included in the multigene data set and third codon positions were weighted 0.94 or less, the HBT was obtained, thus demonstrating how these competing topologies are sensitive to the degree of influence imparted by third-position differences.

Evolutionary Rates

Qiu and Palmer (1999) list several factors that might potentially confound the problem of determining the basalmost land plant lineage: (1) a wide ''evolutionary gap'' (both with morphological and molecular characters) between charophyte outgroups and land plants that eliminates synapomorphies; (2) long-branch attraction problems between ingroup and outgroup; (3) ancient and rapid radiation of bryophyte lineages at the base of the land plant tree, leaving little phylogenetic signal; and (4) extinction. As discussed above, the sequence divergence between *Chara* and a hornwort is equivalent to that between a hornwort and a flowering plant, thus demonstrating that the ''evolutionary gap'' between ingroup and outgroup is not disproportionate, at least for slowly evolving genes such as mtSSU rDNA. As shown in figures 2 and 3, both the gnetophyte and the *Selaginella* clades have increased substitution rates, as indicated by their branch lengths, but in neither case can this be attributed to low taxon sampling. The fast evolutionary rate of gnetophytes has been well documented; however, we do not feel the position of the gnetophyte clade as sister to conifers is artifactual given that this result is in agreement with several recent molecular phylogenetic studies (Bowe, Coat, and dePamphilis 2000; Chaw et al. 2000; Sanderson et al. 2000). Similarly, despite the long branch leading to *Selaginella,* its position as sister to *Isoëtes* when ML+rates was employed is fully in line with molecular and morphological analyses. Long-branch effects can be demonstrated with the nuSSU rDNA partition when analyzed alone with MP. Here, *Selaginella* migrates to the base of the tree, near liverworts. Among bryophytes, there is some evidence of lineage-specific and gene-specific substitution rate heterogeneity. For example, transition rates in complex thalloid liverwort *rbcL* are about half those seen in leafy and simple thalloid liverworts (Lewis, Mishler, and Vilgalys 1997), whereas the opposite is true for nuSSU rDNA (Capesius and Bopp 1997). There is no indication in the present analysis, however, that relationships within and among hornworts, liverworts, and mosses are being influenced by long-branch attraction artifacts.

Mitochondrial Introns

The presence/absence of three mitochondrial introns (Qiu et al. 1998) has been interpreted as supporting the LBT, as all three introns ''are present, with occasional losses, in mosses, hornworts and all major lineages of vascular plants, but are entirely absent from liverworts, green algae and all other eukaryotes.'' How can this result be reconciled with the findings reported here? If the HBT is correct, these introns must have been acquired in the common ancestor of all land plants and

then lost in the common ancestor of liverworts. This scenario is more likely if liverworts are monophyletic, as supported by our analyses (fig. 1), than if they are paraphyletic, as suggested by some previous studies that sampled more broadly within liverworts (Capesius and Bopp 1997; Lewis, Mishler, and Vilgalys 1997). Postulating a single additional loss of each intron is not unreasonable given that all three introns are already known to have been lost in two different lineages (*Isoëtes* and *Ephedra*) and that two of the three introns have been lost many times in land plant evolution (Qiu et al. 1998; Qiu and Palmer, personal communication). Addition of the three intron characters to the multigene matrix does not change the MP−3Ti tree topology, and BS support for the HBT remains high (92%; data not shown).

Reinterpretation of Morphological Features

Given the strong support for the HBT, reinterpretation of key morphological and anatomical innovations is now required. Several ultrastructural features shared between hornworts and charophycean algae (such as *Coleochaete*) support the concept that hornworts are the earliest land plant lineage. Unique among land plants, hornwort chloroplasts contain pyrenoids (localized groupings of RUBISCO) that are essentially identical to those in green algae (Vaughn et al. 1992) and also share with algae channel thylakoids. The HBT thus requires that these chloroplast structures have been lost in other land plants. Conversely, hornworts lack chloroplast granal end membranes and staggered spermatozoid flagella, which presumably evolved subsequent to the divergence of hornworts and other land plants (Garbary and Renzaglia 1998). If the HBT and the sister group status of liverworts and mosses are correct, the presence of stomates in some hornworts and mosses and all vascular plants may indicate that these structures are not homologous (Renzaglia et al. 2000). If moss and vascular plant stomates are homologous, then stomates were lost in the ancestor of liverworts. Similarly, a loss in liverworts is invoked for the perine layer in spores, a feature shared by mosses and vascular plants (Garbary and Renzaglia 1998).

As in most analyses to date, the paraphyly of bryophytes is demonstrated in this multigene analysis. The sister group relationship between mosses and liverworts has received support from data derived from spermatogenesis (Garbary, Renzaglia, and Duckett 1993), sperm ultrastructure (Maden et al. 1997), and overall morphology (Garbary and Renzaglia 1998), as well as molecular characters such as nuclear SSU rDNA (Hedderson, Chapman, and Cox 1998) and mitochondrial SSU rDNA (Duff and Nickrent 1999) and an analysis of five chloroplast genes (Nishiyama and Kato 1999). The morphological and ultrastructural synapomorphies that support a moss/liverwort clade are (1) the presence of gametophytic slime papillae, (2) superficial archegonia, (3) dimorphic basal bodies on spermatozoa, and (4) apertures associated with the spline on spermatozoa (Garbary and Renzaglia 1998). *Takakia* was first described as a liverwort; however, substantial morphological (Renzaglia, McFarland, and Smith 1997) and molecular (Hedderson, Chapman, and Rootes 1996; Duff and Nickrent 1999) evidence now places the genus within the mosses, a result supported by this multigene analysis. Additional sequencing to allow inclusion of putatively basal liverworts such as *Haplomitrium* and *Monoclea* in future multigene analyses is required to test the robustness of the moss-liverwort clade.

Conclusions and Future Work

Although both a thalloid and a leafy liverwort were included in this study, increased sampling of this group and other bryophytes is required to represent more adequately the overall taxonomic diversity of bryophytes; work is currently underway to remedy this situation (unpublished data). In addition to ingroup taxon density, inclusion of other green algal outgroup taxa is critical for future land plant phylogenetic studies. As mentioned in *Materials and Methods*, a mtSSU rDNA sequence for *Coleochaete* has yet to be obtained. When sequences from three genes from *Coleochaete* are included in the four-gene data set (with its mtSSU rDNA scored as missing), an MP+3Ti analysis yields the HBT (73% BS support) as opposed to the LBT, which is obtained without these *Coleochaete* sequences. As the taxonomic breadth of sequences increases, other genes, such as mitochondrial *nad5* (Beckert et al. 1999), *cox3* (Malek et al. 1996), and chloroplast photosystem genes (Nishiyama and Kato 1999), can be added to the present four-gene data set. A preliminary analysis in which *nad5* was added to the four-gene matrix reported here (18 ingroup taxa, half of which were placeholders) gave a tree fully congruent with the one shown in figure 3, with the HBT receiving 90% BS support (unpublished data). The present study, and several recent ones on angiosperm and gymnosperm evolution, demonstrate the tremendous utility of mitochondrial genes. That these genes often give strong support for many noncontroversial relationships provides a compelling reason to consider seriously those relationships inferred from these genes that are not recovered using other data sets.

As stated in the *Introduction*, a goal of this study was to determine whether molecular and morphological data better support the LBT or the HBT. It appears that most support for the former hypothesis derives from the chloroplast gene *rbcL*, specifically, third-codon-position transitions of this gene. In contrast, the HBT is supported by the present study, as well as combined analyses of *rbcL* and other chloroplast genes (Nishiyama and Kato 1999), mitochondrial genes (Malek et al. 1996; Beckert et al. 1999; Duff and Nickrent 1999), nuclear SSU rDNA (Hedderson, Chapman, and Cox 1998), and morphology (Garbary and Renzaglia 1998). Decay analyses indicate that all the alternative topologies tested using MP result in longer trees, and K-H tests show that these alternatives, including the LBT, are statistically different from the HBT. Thus, we conclude that the HBT hypothesis is most consistent with all of the available evidence (morphological and molecular), is not in conflict with presence/ab-

sence data on mitochondrial introns, and therefore provides the best explanation of early land plant evolution. Further refinement and resolution of this tree will provide a firm phylogenetic foundation to help interpret the evolution of plant morphology, ultrastructure, development, and biochemistry.

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