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A Molecular Approach to the Phylogeny of Bryophytes: Cladistic Analysis of Chloroplast-Encoded 16S and 23S Ribosomal RNA Genes

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Abstract. *As the most primitive living lineages of embryophytes, bryophytes are critical to an understanding of the evolution of the land flora. A relatively robust phylogenetic hypothesis exists, based on morphology, ultrastructure, and chemistry, in which the classical group "bryophytes" is not monophyletic. Instead, the mosses seem to be more closely related to the tracheophytes than to the hornworts or liverworts. However, details of these relationships remain unclear. In this paper we explore the usefulness of comparative molecular studies as a potential source of independent data to test and refine this cladogram. We have generated preliminary nucleotide sequence data from portions of the 16S and 23S ribosomal RNA genes in the chloroplasts of 11 bryophytes selected from diverse groups. These data, even though they show considerable homoplasy, appear to be historically informative at this deep phylogenetic level since they support a cladogram that is identical to the morphological one. Future studies will expand the molecular comparisons by sequencing the rest of these two rRNA genes, as well as the protein-coding gene* rbc*L, for many more species.*

The bryophytes occupy a critical phylogenetic position in our understanding of the origin of the land flora. Their phylogeny has attracted interest over a long period of time (Bower 1935; Campbell 1905; Campbell 1971; Haskell 1949; Khanna 1965; Miller 1974, 1982; Schofield 1985). This interest stems from the fact that they appear to be the most basal lineages among extant land plants, and because of the fundamental diversity among the major groups of bryophytes, which has called into question the monophyly of the bryophytes or even of the land plants (i.e., embryophytes; see Crandall-Stotler 1980, 1984; Duckett & Renzaglia 1988). Much research has focused on bryophyte phylogeny; a number of different character systems have been studied in morphological, anatomical, and developmental detail (reviewed by Berthier 1972; Crandall-Stotler 1981; Frey 1971, 1981; Hébant 1977; Ligrone & Gambardella 1988; Lorch 1931; Renzaglia 1978; Schuster 1984*a,b,c*; Vitt 1984). Early light microscopic research concentrated on morphology, apical meristem segmentation patterns, and sexuality. More recently, ultrastructural investigations have also provided significant data (Brown & Lemmon 1988, 1990; Carothers & Rushing 1988; Duckett et al. 1983; Duckett & Renzaglia 1988; Scheirer 1980).

Critical evolutionary issues in the origin and radiation of the land plants include the nature of reproduction, source of the alternation of generations, and water relationships (Graham 1985; Mishler & Churchill 1984, 1985). Such evolutionary questions can best be addressed through comparative methods that are based on reconstructions of character evolution on cladograms (Brandon 1990; Brooks & McLennan 1991; Coddington 1988; Donoghue 1989; Harvey & Pagel 1991; Maddison 1990; Mishler 1988; Ridley 1983). Such methods obviously depend on the quality of the reference cladistic topology, that should be based on many characters in addition to (or ideally with the exclusion of) the specific characters of evolutionary interest. Therefore, the time seems ripe to add molecular characters to the data base.

The status of available phylogenetic characters was first analyzed cladistically by Mishler and Churchill (1984, 1985; see also Bremer et al. 1987; Graham et al. 1991; and Mishler 1986). The current hypothesis of land plant relationships based on these studies can be outlined as follows. The embryophytes (land plants, which include the bryophytes and tracheophytes), are well supported as a monophyletic group by several synapomorphies. Closely related sister groups of the embryophytes are a number of lineages of green algae that have been lumped together in the paraphyletic group "charophytes" (Mattox & Stewart 1984; Stewart & Mattox 1975). Among these, the immediate living sister group of the embryophytes appears to be the genus *Coleochaete* alone. Within the embryophytes, the classical inclusive group "bryophytes" also appears to be paraphyletic. The mosses alone are the sister group of the tracheophytes (the so-called "vascular plants"),

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as evidenced by a number of synapomorphies, putatively including xylem and phloem (Mishler & Churchill 1984). Currently, the hornworts are best placed as the sister group of the moss–tracheophyte clade, but this placement is problematical because of the number of homoplasies involving the hornworts. Finally, the liverworts are the sister group of the other extant land plants.

Considerable interest has recently been focused on tapping the historical information that resides in DNA. Large portions of the genome are quite conservative over long evolutionary times, and have proved useful in resolving relationships in a number of different cases, including bacteria, fungi, plants, birds, and primates (e.g., see volumes edited by Fernholm et al. 1989; Hillis & Moritz 1990; Soltis et al. 1992). Chloroplasts of green plants have their own DNA, quite distinct from that of the nucleus (due to the ancient origin of the chloroplast from an endosymbiotic, photosynthetic bacterium). Chloroplast DNA has proven to be especially conservative over evolutionary time, and has been useful for phylogenetic studies in the flowering plants (Jansen & Palmer 1988; Palmer 1985; Palmer et al. 1988; Sytsma & Gottlieb 1986). Current understanding of chloroplast DNA evolution was reviewed by Birky (1988) and Zurawski and Clegg (1987).

The work of a number of researchers on structural aspects of the chloroplast genome (reviewed by Palmer 1985) has led to the elucidation of a consensus gene order for the angiosperms that is highly conserved evolutionarily. Two inverted repeat regions (containing the ribosomal RNA genes) are separated by a large and a small single copy region. With only slight modification, this consensus gene order has also been found in a liverwort, *Marchantia polymorpha* (Ohyama et al. 1983, 1986), in a fern, *Osmunda cinnamomea* (Palmer & Stein 1982) and in a moss, *Physcomitrella patens* (Calie & Hughes 1987).

The consensus structure of the land plant chloroplast genome provides additional strong evidence for monophyly of the group, given that the structure of the chloroplast genome in closely related "charophyte" green algal outgroups is quite different (J. R. Manhart, pers. comm.). Chloroplast studies have also confirmed the close relationship of *Coleochaete* to the land plants (Baldauf & Palmer 1990; Baldauf et al. 1990; Manhart & Palmer 1990).

Calie and Hughes (1987) showed that the chloroplast genome of the moss *Physcomitrella* has two interesting exceptions to the general order and arrangement of the consensus land plant genome. First, the *rpl*2 and *rps*19 genes are shifted from a position adjacent to the *petB/petD* gene cluster to a position next to the *atpH* gene. Second (and potentially more important), the *atpH* and *psbA* genes have exchanged positions; the significance being that the moss *Physcomitrella* shares this arrangement with the liverwort *Marchantia*. This finding is in need of polarization by outgroup comparison since it could represent either a synapomorphy or a symplesiomorphy (previously available data indicate that mosses are more closely related to the tracheophytes than to the liverworts).

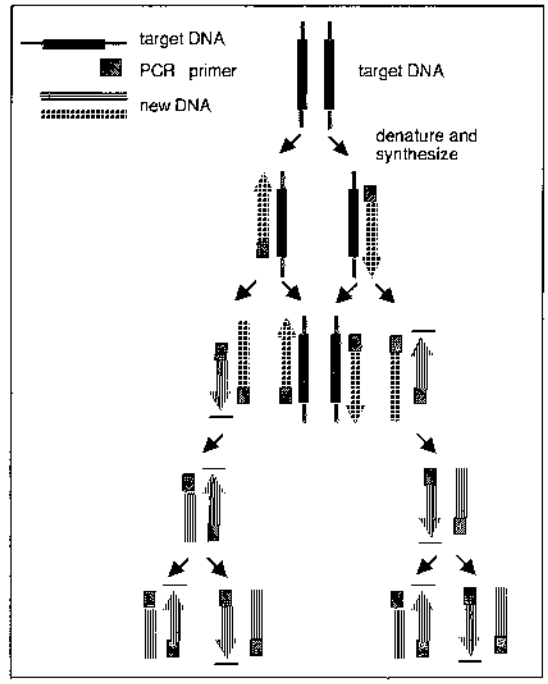


FIGURE 1. The polymerase chain reaction (PCR). Primers are based on known, conserved sequences from related organisms. A pair of primers (one forward, one reverse) is mixed with bulk DNA extracted from a target organism and solutions of extra deoxynucleotides (of all four types: A, C, G & T) in the presence of a thermally-stable DNA polymerase (AmpliTaq). Repeated cycles (20 to 30) of denaturation, primer annealing, and DNA synthesis are carried out in a thermal-cycling waterbath, resulting in an approximately two-fold increase in the target sequence per cycle (based on descriptive material distributed by Perkin-Elmer Cetus).

In this paper, we report partial nucleotide sequences of two chloroplast-encoded ribosomal RNA genes (16S and 23S). These genes are reputed to be among the slowest-evolving in the chloroplast genome (i.e., denoted as "superslow" by Palmer, unpubl. data), and thus were thought to be potentially informative about ancient branching events in early land plant diversification. Our goal was two-fold: *1)* to generate a preliminary data set that could be used to test the suitability of these genes for phylogenetic reconstruction at this "deep" level; *2)* to demonstrate new methods of DNA sequencing that

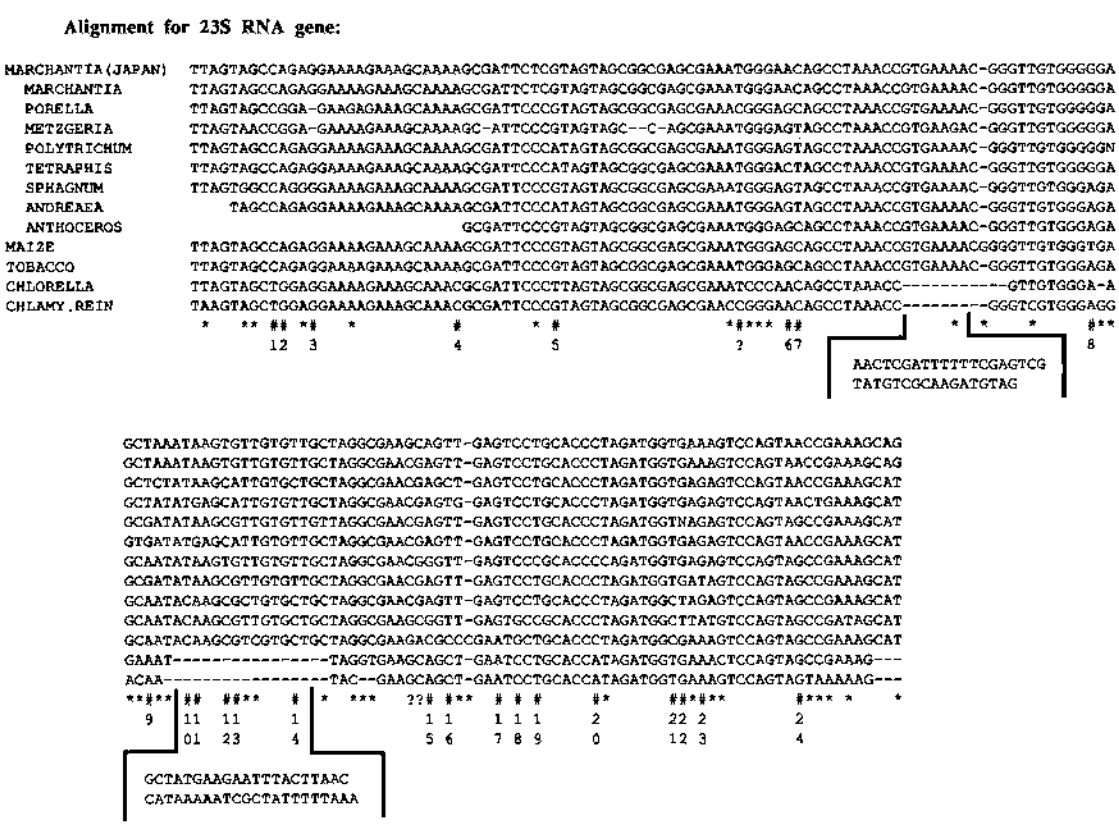


FIGURE 2. Aligned partial sequences for the chloroplast-encoded 23S rRNA gene, for eight study taxa (names indented) and five taxa taken from GENBANK. Gaps are shown by "–"; unreadable bases are shown by "N"; regions where the two green algal outgroups were unalignable with the land plants are set off below. Autapomorphous differences are marked "*"; taxonomically informative differences are marked "#" and numbered. Three positions were potentially informative, but were rejected (marked with a question mark): the position before #6 because the algae are questionably alignable here, and the two positions before #15 because the sequence read by us in all cases (CG) was inverted relative to all the sequences from GENBANK, even the *Marchantia* sequence (we suspect a gel reading problem).

have the potential to be widely useful in bryophyte systematics.

MATERIALS AND METHODS

Total genomic DNA was extracted from 11 species of bryophytes (listed below) using a standard hot CETAB procedure (Doyle & Dickson 1987). Nine samples were from field-collected populations and two were from herbarium specimens [that yielded perfectly satisfactory DNA for polymerase chain reaction amplification (PCR, see Fig. 1) from only a few stems]. The *Marchantia* population from North Carolina was included, even though the entire chloroplast genome has been sequenced in a Japanese population of the same species (Ohyama et al. 1986), to verify our methods. All samples were washed thoroughly and checked carefully with a dissecting microscope to remove any contaminating plants.

Populations studied (all from North Carolina, with voucher specimens deposited at DUKE): *Andreaea rothii* Web. & Mohr. STOKES CO.: *Anderson 24716*, 6/5/86 (DNA extracted from herbarium specimen during 5/89). *Anthoceros punctatus* L. DURHAM CO.: *Mishler et al.*, 5/9/89 (only sporophytes were used, to avoid contamination problems with closely associated plants). *Conocephalum conicum* (L.) Lindb. ORANGE CO.: *Mishler et al.*, 5/8/89. *Marchantia polymorpha* L. DURHAM CO.: *Mishler*, 5/9/89. *Metzgeria furcata* (L.) Dum. ORANGE CO.: *Mishler et al.*, 5/9/89. *Polytrichum commune* Hedw. DURHAM CO.: *Mishler et al.*, 5/9/89. *Porella pinnata* L. ORANGE CO.: *Mishler et al.*, 5/8/89. *Sphaerocarpos texanus* Aust. DURHAM CO.: *Schwartz*, 5/5/89. *Sphagnum compactum* DC. *in* Lam. & DC. DURHAM CO.: *Mishler et al.*, 5/9/89. *Tetraphis pellucida* Hedw. BUNCOMBE CO.: *Pittillo 9764*, 7/8/88 (DNA extracted from herbarium specimen during 5/89). *Thuidium delicatulum* (Hedw.) B.S.G. MCDOWELL CO.: *Newton*, 11/18/88.

Oligonucleotide primers for amplification and sequencing of cpDNA were designed based on comparisons among previously-known conserved sequences in *Chlamydomonas*, *Marchantia*, and tobacco. These primers were in pairs about 250 nucleotides apart, one pair near the beginning of the 16S RNA gene (primers 16S1: 5'-GAACGCTGGCGGCATGC and 16S2R: 5'-AGTGTGGCTGATCATCC) and the other pair near the beginning of the 23S RNA gene (primers 23S1: 5'-GCAGGCAAGAGACAACC and 23S2R: 5'-CTTCAACAGGCACGCGG).

Direct sequencing of PCR products was performed using the kinase method described by Higuchi and Ochman (1989). For each PCR reaction, the forward primer (either 16S1 or 23S1) was tagged with a terminal phosphate on

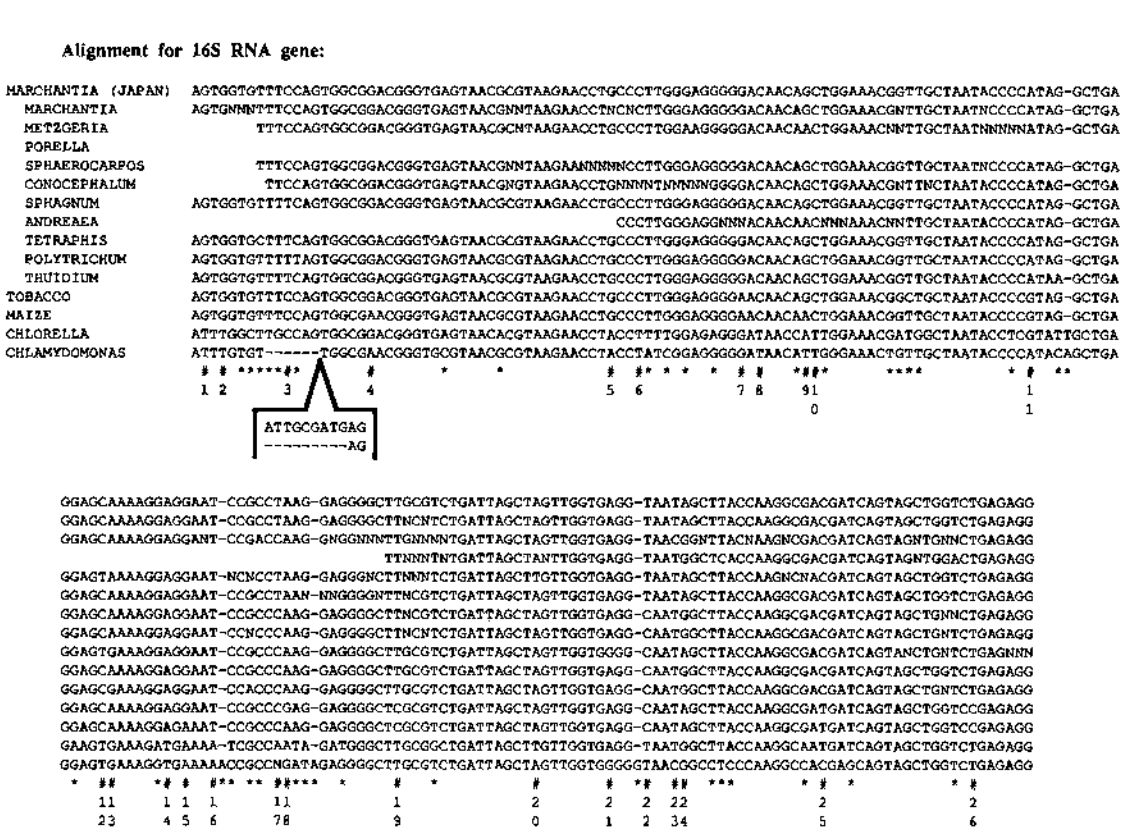


FIGURE 3. Aligned partial sequences for the chloroplast-encoded 16S rRNA gene, for ten study taxa (names indented) and five taxa taken from GENBANK. Gaps are shown by "–"; unreadable bases are shown by "N"; a region where the two green algal outgroups were unalignable with the land plants is set off below. Autapomorphous differences are marked "*"; taxonomically informative differences are marked "#" and numbered.

its 5′ end using DNA kinase. Standard buffer conditions and Taq polymerase (AmpliTaq) for PCR were used according to manufacturer's suggestions (Perkin-Elmer Cetus Corp.). After amplification, the kinased strand from each amplified DNA was digested using lambda exonuclease, leaving single-stranded templates suitable for sequence analysis. Sequencing was then performed using the forward primer following the recommended protocol for Sequenase (U.S. Biochemical). Additional details regarding these primers and the protocols used for PCR and sequencing are available from the authors on request.

Sequence data entry and initial alignments were done using the MicroGenie computer package (vers. 4, Beckman Instruments), and adjusted by eye. Only unambiguously alignable regions were used for phylogenetic analysis; highly variable regions were ignored. Potentially informative characters were analyzed using the branch and bound algorithm in PAUP (version 3.0r for the Macintosh; Swofford 1991); all characters were unordered and the two algal taxa were designated as outgroups. The two data sets were analyzed separately and also together as a combined data set (in the last case using only the seven species for which sequences from both genes were obtained).

RESULTS

For the 23S gene we were able to sequence about 170 nucleotides for eight species (see Fig. 2); for the 16S gene we were able to sequence about 200 nucleotides for 10 species (see Fig. 3). The data for both genes were aligned with previously-known sequences from *Chlamydomonas* and *Chlorella* (green algae used as outgroups), the "known" *Marchantia* sequence from Japan, and corn and tobacco (used to represent tracheophytes within the ingroup). Pairwise identities between bryophytes were quite high for both genes, e.g., *Marchantia* and *Polytrichum* were about 95% identical for the 23S sequence and 97% identical for the 16S sequence. There were 24 potentially informative characters discovered in the 23S data, and 26 in the 16S data (marked in Fig. 2 and 3).

The 23S data set, analyzed alone, gives eight equally parsimonious trees [length = 46; consistency index (CI) = 0.652], the consensus of which (Fig. 4) is completely unresolved except for two components: the land plants and *Porella–Metzgeria*. The 16S data set analyzed alone gives five equally parsimonious trees (length = 42; CI = 0.667), the consensus of which (Fig. 5) is more resolved, but clearly untenable with respect to previous ideas of relationship: the thalloid hepatics alone are shown as

FIGURE 4.
23S Alone

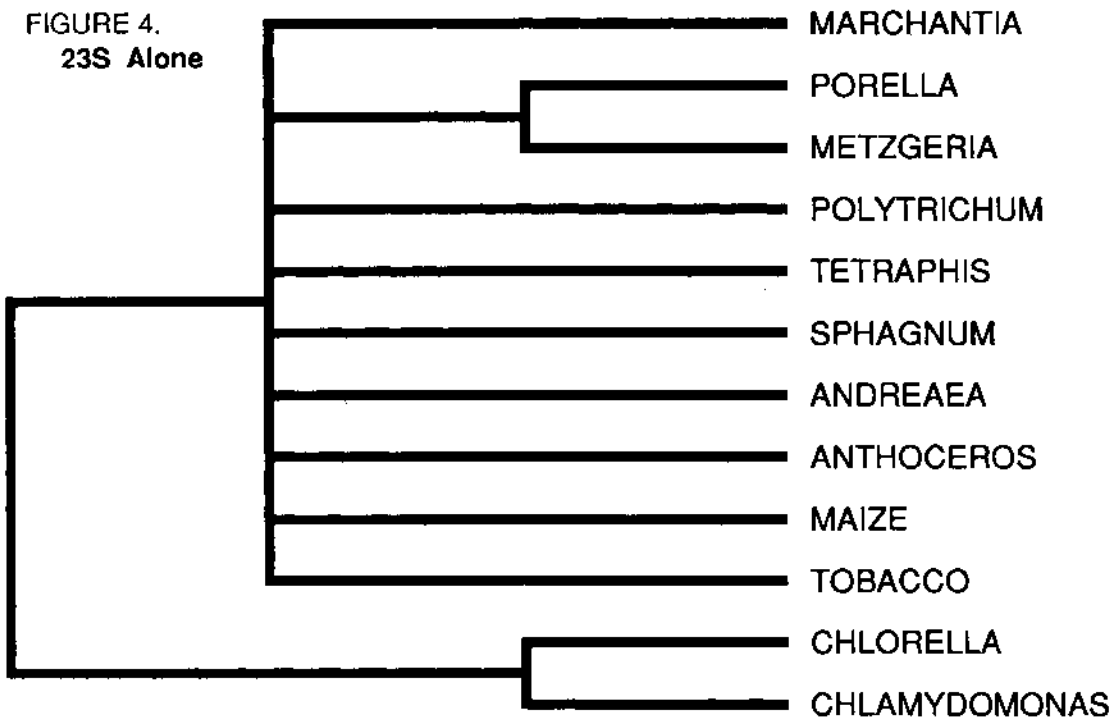
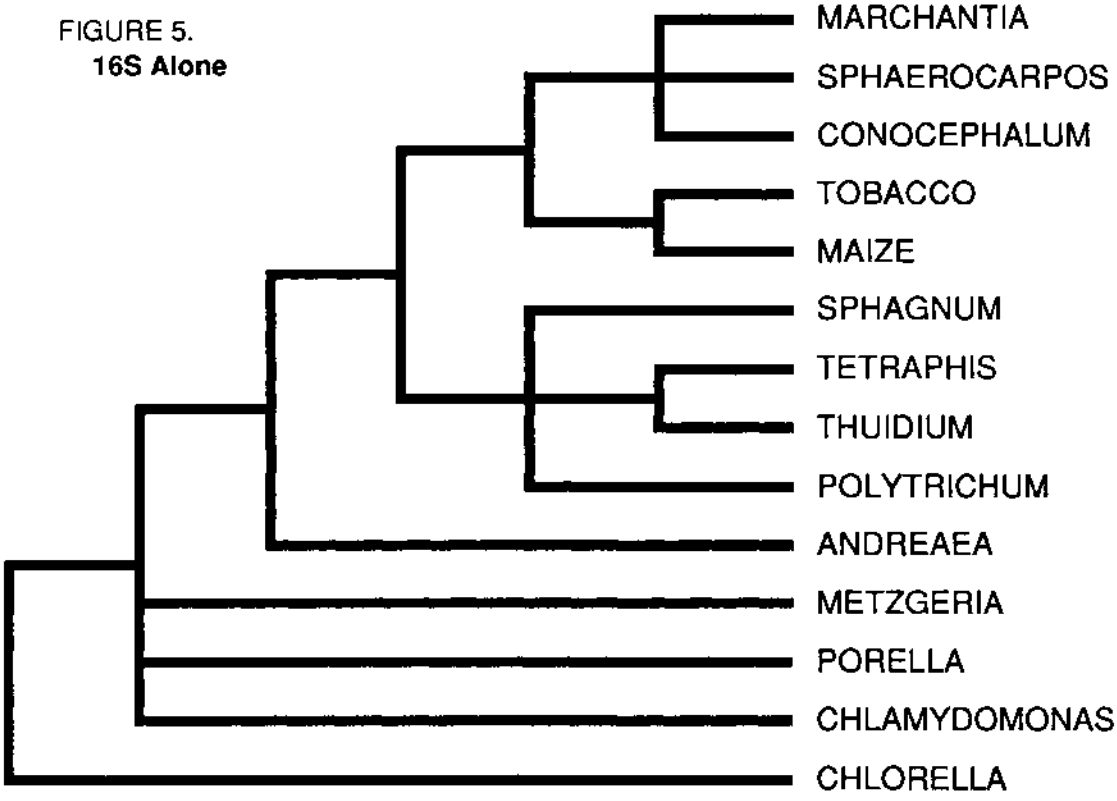


FIGURE 5.
16S Alone



FIGURES 4–5. Strict consensus trees from separate parsimony analyses of sequence data from chloroplast-encoded 23S rRNA and 16S rRNA genes.

FIGURE 6.
Combined Data

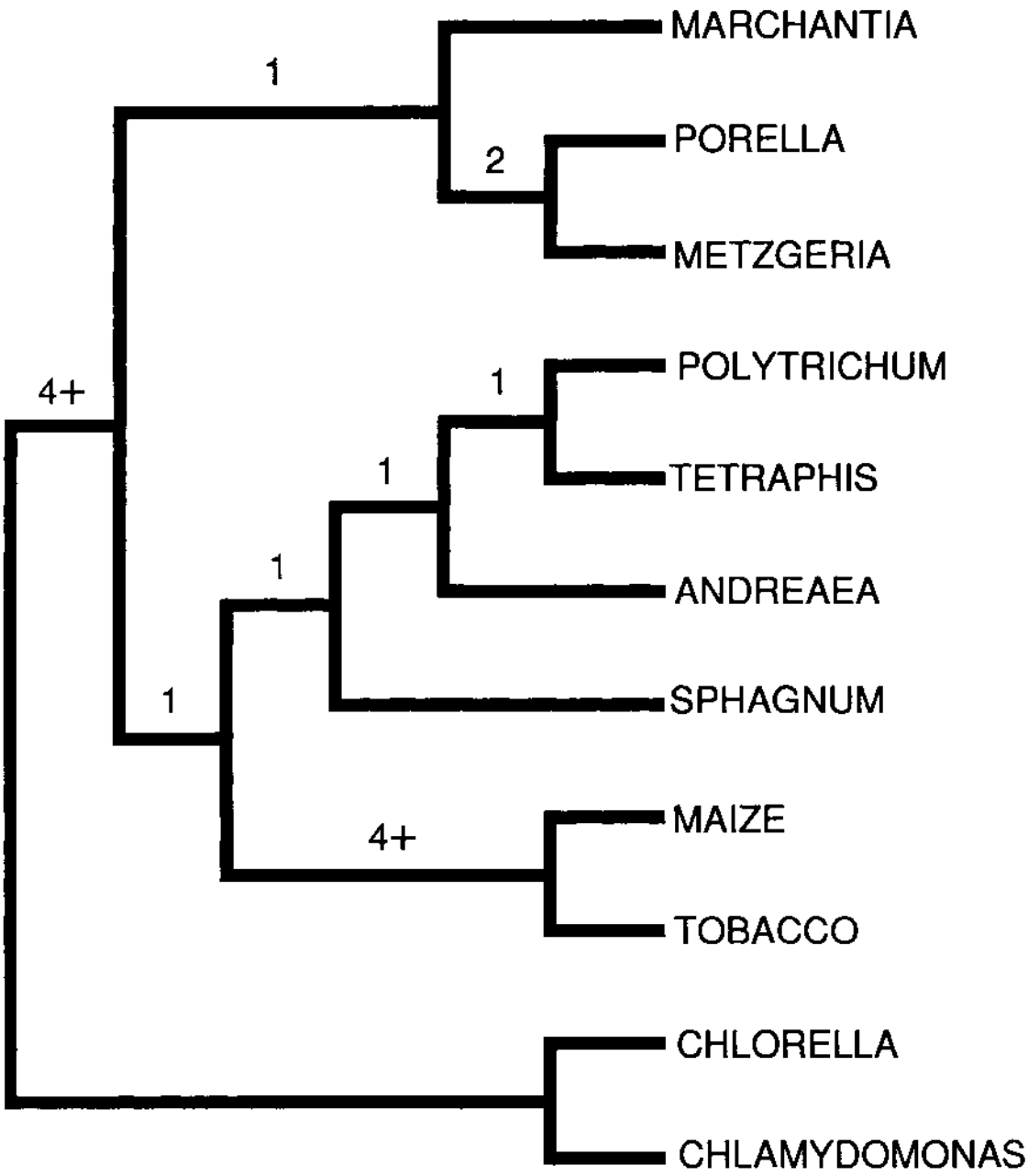


FIGURE 6. Cladogram based on a parsimony analysis of combined sequence data from chloroplast-encoded 16S rRNA and 23S rRNA genes of seven bryophytes (with two angiosperm representatives and two green algal outgroups from the literature). The decay index (i.e., the number of steps parsimony must be relaxed to make support for a clade disappear) is given for each internal branch.

the sister group to the tracheophytes, the mosses as a paraphyletic group basal to them, and the leafy hepatics as basal lineages mixed in with the two green algal outgroups.

Despite the lack of consistent resolution between the two data sets when they were analyzed separately, an informative result was obtained by combining them, giving a total of 50 characters for the seven bryophyte species for which sequences of both genes were obtained. The combined data yield a

single most-parsimonious tree (87 steps long, CI = 0.651, Fig. 6) that exactly matches the phylogenetic topology for the same taxa supported by the morphological data (see Mishler 1986): the mosses appear as a monophyletic sister group to the tracheophytes, and the liverworts as the sister group to the moss–tracheophyte lineage; the branching order within the mosses and liverworts also matches.

Discussion

Nucleic acid sequence data from any particular gene might be expected to be phylogenetically informative only through some "window" of divergence time (Mishler et al. 1988); the restricted number of character states (four) leads to problems of "mistaken identity" (i.e., nonhomologous matches) when multiple mutations have occurred at a site. Thus, molecular data are no panacea for phylogeny reconstruction (contrary to the opinion of many authors, e.g., Duckett & Renzaglia 1988). The gene to be sequenced must be chosen carefully based on preliminary studies that indicate feasibility (much in the way that more traditional character systems are evaluated), and analyzed properly (using a method designed to "extract" phylogenetic information optimally). We have attempted in this paper to address both considerations for the two chloroplast-encoded rRNA genes at the phylogenetic level of the major groups of land plants.

Our molecular data show a relatively high level of homoplasy (as compared to the available morphological data for these plants). However, the consistency index for the parsimony analyses, ca. 0.65–0.70, is typical of many other higher-level phylogenetic analyses with this number of taxa (Donoghue & Sanderson 1992). Our results suggest that the combined sequence data do contain historical information at this phylogenetic level (the exact match with the morphological topology is very improbable under any random model—there are over 600 million possible bifurcating trees with 11 OTUs!), but the topology is not particularly robust. Examination of slightly less parsimonious trees is the basis of a sensitive measure of relative support for clades known as the "decay index" (Mishler, Donoghue & Albert, unpubl. data), i.e., the number of steps that parsimony must be relaxed to remove support for a particular node. Such an examination of the combined data shows that the interesting structure of Figure 6 "decays" fast; the consensus with trees one step longer (i.e., $\leq$ 88 steps) is mostly unresolved, with *Metzgeria–Porella* and tobacco–maize the only resolved clades; the consensus with trees two and three steps longer (i.e., $\leq$ 90 steps) retains only the angiosperm clade.

The only other nucleotide sequence data set previously available for bryophytes is from nuclear-encoded 5S rRNA (Hori et al. 1985; Hori & Osawa 1987). The limitations of these data were discussed by Bremer et al. (1987), Mishler et al. (1988), and Steele et al. (1991). The basic problem with this molecule from a phylogenetic standpoint seems to be its small size (only 120 nucleotides long) and uneven evolutionary constraints. Large parts of the molecule are invariant, and the sites that do vary appear to be saturated with multiple mutations resulting in homoplasy and poor topological resolution.

The 5S rRNA data set illustrates the importance of the selection of methods for systematic analysis. The realization of difficulties with those data was only possible from a cladistic standpoint; the Hori papers presented phenograms (i.e., branching diagrams based on distance measures) which obscured the real conflict in the data (criticisms of phenetic approaches to phylogeny reconstruction are given by Farris 1982). The cladistic approach takes into account the evidential meaning of each individual putative homology (rather than combining all homologies into a single distance statistic with considerable loss of information). The branching diagram that retains the maximum number of the original, independently specified hypotheses of homology is to be preferred under the parsimony criterion used in cladistic analysis (see Farris 1983, Mishler & De Luna 1991, and Sober 1988 for detailed discussion and justification of the cladistic method and its assumptions). The cladistic approach has become standard in plant molecular systematics, along with a greater appreciation of potential problems with phylogenetic analysis of molecular data (Albert et al. 1992; Bremer 1988; Donoghue & Sanderson 1992; Hillis 1987; Mishler et al. 1988; Steele et al. 1988; Sytsma 1990; Waters et al. 1992).

Our results clearly indicate the desirability of further sequencing of chloroplast DNA. The beneficial effect of combining the two preliminary data sets may be due either simply to the effect of adding more characters to the analysis or (perhaps more interestingly) to a sort of mutual support of the data sets wherein one data set provides support for clades that the other does not provide. This possibility is currently being tested in our laboratory by more extensive sequencing of the 16S and 23S rRNA genes, as well as the protein-coding gene *rbc*L, from the chloroplasts of many other species of bryophytes.

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