ABSTRACT

Title of dissertation: PHYLOGENETIC STUDIES OF THE CHARALES: THE CLOSEST LIVING RELATIVES OF LAND PLANTS

Kenneth Gregory Karol, Doctor of Philosophy, 2004

Dissertation directed by: Associate Professor Charles F. Delwiche

Department of Plant Biology

The embryophytes (land plants) consist of organisms such as mosses, ferns, conifers and flowering plants. Although land plants have long been thought to be related to the green algal group Charophyceae, the nature of this relationship has been unresolved for over a century. A four-gene phylogenetic analysis supports the hypothesis that land plants arose from within the Charophyta and unambiguously identifies the Charales as the closest living relatives of plants. With a robust phylogeny, it is now possibly to explore phylogeny-dependant questions that were previously difficult to assess. Estimating the divergence time of the land plant lineage is one such question. A recent time estimate for the colonization of land by plants is 1,061 ± 109 mya and 703 ± 45 mya for the divergence of vascular plants and bryophytes, a result much older than the

fossil record suggests (roughly 470 mya). Unlike most algae, a rich fossil record exists for the Charales in the form of calcified oospores. Representative fossils that can be attributed to five extant lineages in the Charales have been identified with reasonable accuracy. These multiple calibration points were used in conjunction with the four-gene DNA data set to estimate the divergence time of the land plant and Charales lineages. The Bayesian relaxed-clock approach estimated divergence of the Charales/land plant common ancestor in the Late Proterozoic (674.10 ± 99.96 MYA), modern land plants in the Cambrian (497.78 ± 75.66 MYA), and modern Characeae at the Paleozoic/Mesozoic boundary (247.75 ± 25.98 MYA). The genus *Nitella* is one of the most diverse genera in the Charales. Wood and Imahori's worldwide monograph divides *Nitella* into three subgenera with seventeen sections and radically modified the taxonomy of this group my submerging over 200 *Nitella* species into 53 loosely defined species. Phylogenetic analyses of *rbcL* sequence data from 79 *Nitella* species (plus outgroups) support the monophyly of *Nitella* and two subgenera (Hyella and Tieffallenia). Subgenus Nitella formed two paraphyletic lineages at the base of the genus. Few sections were monophyletic and species diversity is interpreted as being much higher than proposed by Wood.

PHYLOGENETIC STUDIES OF THE CHARALES: THE CLOSEST LIVING RELATIVES OF LAND PLANTS

by

Kenneth Gregory Karol

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Advisory Committee:

Associate Professor Charles F. Delwiche, Chair
Professor Todd J. Cooke
Dr. Richard M. McCourt
Professor Charles Mitter
Dr. Elizabeth A. Zimmer

To Gregory A. French

Acknowledgements

As an undergraduate student at the University of Madison I distinctly remember asking a group of graduate students "What does a Ph.D mean?" After some discussion, the group converged on the answer that a Ph.D. demonstrates a students' ability to 'perform independent research at a high academic level.' Now that I have been through this process I must disagree. If it weren't for the assistance and support of numerous friends and colleagues this dissertation would have been impossible.

I am particularly indebted to my graduate committee. Chuck Delwiche for offering the opportunity to be his student and supplying the environment for brutally honest scientific discussion and the freedom to think out loud. Todd Cook for recognizing the more unusual side of my personality and allowing it to grow, not to mention stimulating conversations in the halls and on the loading dock. Rick McCourt for his never-ending friendship and for introducing me to the Characeae. Liz Zimmer for her encouragement, friendship and scientific integrity and Charlie Mitter for keeping me on my toes.

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Introduction

Overview of the Charophyta

Green plants *sensu lato* are comprised of two distinct lineages termed Charophyta and Chlorophyta. The Charophyta represent an important lineage in the tree of life because land plants (or embryophytes) are derived from within this group and therefore have received considerable attention since the early history of systematic botany (Bower, 1908; Darwin, 1859; Fritsch, 1935; Smith, 1938). The Charophyta comprise not only the land plants but also several lineages of fresh-water green algae. The latter exhibit a broad range of morphological diversity including biflagellate unicells (Mesostigmatales), sessile unicells (some Zygnematales), sarcinoid packets of cells (Chlorokybales), unbranched filaments (Klebsormidiales and some Zygnematales), and relatively complex branched filaments (Charales and Coleochaetales). The Charophyta are sister to the Chlorophyta, which comprises essentially all other green algae (Friedl, 1997; Mattox and Stewart, 1984; Mishler et al., 1994; Pickett-Heaps, 1975; Pickett-Heaps and Marchant, 1972).

Several influential works emphasized ultrastructural features such as mode of cell division and flagellar root structure to erect the green algal class Charophyceae (Mattox and Stewart, 1984; Pickett-Heaps, 1975; Stewart and Mattox, 1975; Stewart and Mattox, 1978). A suite of biochemical and additional ultrastructural characters have since been identified that confirm a close relationship of these algae with land plants (Cook and Graham, 1998; Cook et al., 1997; Cook et al., 1998; De Jesus et al., 1989; Delwiche et

al., 1989; Domozych et al., 1980; Frederick et al., 1973; Hotchkiss Jr. and Brown Jr., 1987; Jacobshagen and Schnarrenberger, 1990; Okuda and Brown Jr., 1992; Syrett and Al-Houty, 1984). Molecular phylogenetic analyses have verified monophyly of most of the charophyte orders and unequivocally established land plant ancestry within the charophycean green algae (Chapman and Buchheim, 1991; Chapman et al., 1998; Delwiche et al., 2002; Delwiche et al., 1995; Gontcharov et al., 2003; Manhart, 1994; Marin and Melkonian, 1999; McCourt, 1995; McCourt et al., 2000; McCourt et al., 1996a). However, branching patterns among these lineages have been only weakly supported, as measured by bootstrap values (McCourt, 1995). Similarly, morphological and structural genomic data have clarified some relationships (Graham et al., 1991; Manhart and Palmer, 1990; Mishler et al., 1994; Qiu et al., 1998; Sluiman, 1985), but have been limited by uncertain homology assessment and a limited number of characters. Phylogenetic relationships among these lineages have been difficult to resolve with certainty. In particular, identification of the sister taxon to land plants has been problematic.

Overview of the Charales

Commonly called stoneworts or brittleworts, the extant Charales are an evolutionarily important member of the Charophyta. These algae not only share a close evolutionary history with land plants but also have a rich fossil record unique among the charophyte algae (Feist and Grambast-Fessard, 1991; Grambast, 1974; Peck, 1953; Tappan, 1980). Natural historians have been attracted to the Charales since the time of Plinius (1469) and stoneworts later were clearly diagnosed in the herbal of Bauhin

(1623), as *Equisetum foetidum sub aqua repens* (e.g., as a variety of embryophyte). Vaillant (1721) assigned them to a new genus, *Chara*, which was later retained by Linnaeus (1753), who recognized four species. Currently, the Charales contain one extant family, the Characeae, in two tribes with six genera: Chareae (*Chara, Lamprothamnium, Lychnothamnus, Nitellopsis*) and Nitelleae (*Nitella, Tolypella*) (Wood, 1965). *Chara* and *Nitella* are relatively species-rich, while the remaining genera contain only a few (*Lamprothamnium* and *Tolypella*) or a single species (*Lychnothamnus* and *Nitellopsis*).

The Charales play a key ecological role in fresh-water streams, ponds, and lakes throughout the world on all continents except Antarctica (Hutchinson, 1975; Wood, 1965). They are often the first macrophytic vegetation to occupy new or recently inundated freshwater bodies (Crawford, 1977; Guerlesquin, 1991; Keiner, 1944; Wade, 1990; Wood, 1952), and sometimes display complex species depth zonation (Blindow, 1992; Corillion, 1957; de Winton et al., 1991; Hutchinson, 1975; Schwarz et al., 2002; Wood, 1950a). Stoneworts often form a significant part of the submerged vegetation stabilizing the sediment (van Nes et al., 2002) and providing food and shelter for other aquatic organisms (Noordhuis et al., 2002). While typically found at depths less than 10 meters (Round, 1981), the Charales are often the deepest inhabitants of clear lakes (Dale, 1986; de Winton et al., 1991; Guerlesquin, 1991; Spence and Crystal, 1970; Starling et al., 1974; Vant et al., 1986) and have been found growing as deep as 60 meters.

The Charales and their extinct relatives left behind a rich and diverse fossil record, unmatched among the charophyte algae (Feist and Grambast-Fessard, 1991; Grambast, 1974; Peck, 1953; Tappan, 1980). This record traces back to the Upper

Silurian and includes the earliest known bisexual plant (Feist and Feist, 1997). Charophyte fossils consist predominantly of female gametangia (oogonia) that when fossilized are called gyrogonites. Though relatively rare, fossilized antheridia and vegetative thalli have been reported (Martín-Closas, 1999; Martín-Closas and Diéguez, 1998; Taylor et al., 1992). In addition to the Charales, two extinct orders are recognized, the Sycidiales and Trochiliscales. Each order is easily identified by the orientation of sterile jacketing-cell impressions surrounding the gyrogonite [Sycidiales = vertical, Trochiliscales = dextral, and Charales = sinistral (Feist and Grambast-Fessard, 1991; Grambast, 1974; Peck, 1953; Tappan, 1980)]. Three families are currently recognized in the Sycidiales, two in the Trochiliscales and six in the Charales (Feist and Grambast-Fessard, 1991).

The Charales span over 390 million years of evolution from the Middle Devonian to present and include the only extant family (Characeae). *Eochara wickendeni* Choquette from the Middle Devonian (391-370 mya) is the earliest known representative of the Charales and the only know example of the family Eocharaceae (Choquette, 1956). *Paleochara acadica* (Paleocharaceae) from the Pennsylvanian (323-290 mya) is the single representative of its family, being described from only six specimens from one location (Bell, 1922). The Eocharaceae and Paleocharaceae are unique in the Charales in that they exhibit more than six and just six spiral jacket cells, respectively. Jacket cell number in the remaining families, including extant members, is fixed at five. The Porocharaceae are first seen in the Late Carboniferous (Pennsylvanian, 323-290 mya) and the Clavatoraceae appeared in the Late Jurassic (Tithonian, 151-144 mya). Both of these families extend to the Late Cretaceous (Maastrichtian, 71.3-65 mya) after which they are

no longer found. At this time, the Raskyellaceae first appear and extend to the Late Oligocene (Chattian, 28.5-23.8 mya). *Aclistochara* from the Late Triassic is the oldest representative of the Characeae (Liu and Chen, 1992). The Characeae reached its greatest generic diversity during the Eocene and Oligocene; however, diversity declined rapidly in the Miocene eventually stabilizing only six extant genera (Feist and Grambast-Fessard, 1991).

Numerous morphologically based taxonomic schemes that date back to the older botanical literature have been proposed for the Charales (Allen, 1954; Groves and Bullock-Webster, 1920; Groves and Bullock-Webster, 1924; Halsted, 1879; Robinson, 1906; Wood, 1951). A comprehensive monograph is available and widely used (Wood, 1965), in which a large body of diverse work is summarized and a novel taxonomic treatment is presented. This treatment remains controversial after nearly 40 years because it is founded on gross morphological similarity rather than on shared derived characters. Wood (1965) implied that this scheme reflected evolutionary relationships, but Proctor (1980) pointed out that it was derived from a pre-Darwinian classification based on convenience, not on phylogenetic inference. Under this intuitive approach, approximately 400 Characeae species were reduced to intra-specific ranks (i.e., variety or forma) or submerged into synonymy resulting in 81 species (Wood, 1965). This classification has been widely challenged. Results from numerous crossing experiments of *Chara* species (McCracken et al., 1966; Proctor, 1970; Proctor, 1971; Proctor, 1972; Proctor, 1975; Proctor, 1980; Proctor et al., 1971; Proctor and Wiman, 1971) suggest that greater species diversity exists than was proposed in Wood (1965). Moreover, some characters (e.g., stipulodes number, cortication) that were used to delineate groups of

species (sections and subsections) were demonstrated to be environmentally variable (Proctor, 1980). Similarly, diversity within *Nitella,* a relatively ancient and species rich group, remains puzzling and poorly understood. *Nitella* is relatively difficult to culture and crossing experiments are needed.

Several molecular-based phylogenetic studies have been published addressing relationships of genera within the Charales (McCourt et al., 1996a; McCourt et al., 1996b; Meiers et al., 1997; Sanders et al., 2003), and species within *Chara* (McCourt et al., 1999; McCourt et al., in prep; Meiers et al., 1999). Two recent phylogenetic studies of *Nitella* have been published (Sakayama et al., 2004; Sakayama et al., 2002). Detailed SEM images of the oospore membrane were presented and the phylogenetic utility of this character was demonstrated in light of chloroplast gene phylogenies. Taxa in these studies, though valuable, were biogeographically restricted to Japan and Malaysia.

More than 180 species of *Nitella* have been described and Wood (1965) reduced these to just 19, arguing that many of these species represent a 'continuum of morphological characters' and that 'isolated populations have contributed to the complex pattern of morphological variation.' The chloroplast gene phylogenies of Sakayama *et al.*, (2004; 2002) clearly refute this notion and demonstrate that worldwide sampling is greatly needed. With a clearer understanding of evolution within *Nitella*, fundamental classification schemes that are based on natural groups rather than subjective criteria can be constructed. In addition, key innovations or processes within *Nitella* that could help explain not only evolution of *Nitella* but also the origin and diversification of land plants may be identified.

Aims

It is unclear when the lineage leading to land plants diverged from its green algal ancestor or when they successfully colonized and diversified on the land. There is no undisputed fossil of a charophyte older than the Silurian or land plant before the Ordovician (Graham, 1993; Grey et al., 1982; Kenrick and Crane, 1997a). Because primitive plants and most algae do not preserve well in the fossil record, the possibility exists of an earlier, unrecorded history. Consequently, information derived from the fossil record and combined with a robust molecular phylogeny holds strong potential for understanding the order and timing of these events. Members of the Charophyta have been extensively studied as model organisms in such diverse fields as cell biology, conservation, ecology, molecular biology, and paleobotany. Systematic studies (in particular phylogenetic trees) provide fundamental historic data that can be used in virtually all fields of biology. They can serve to unite seemingly disparate areas of biology towards a better evolutionary understanding of this important eukaryotic lineage.

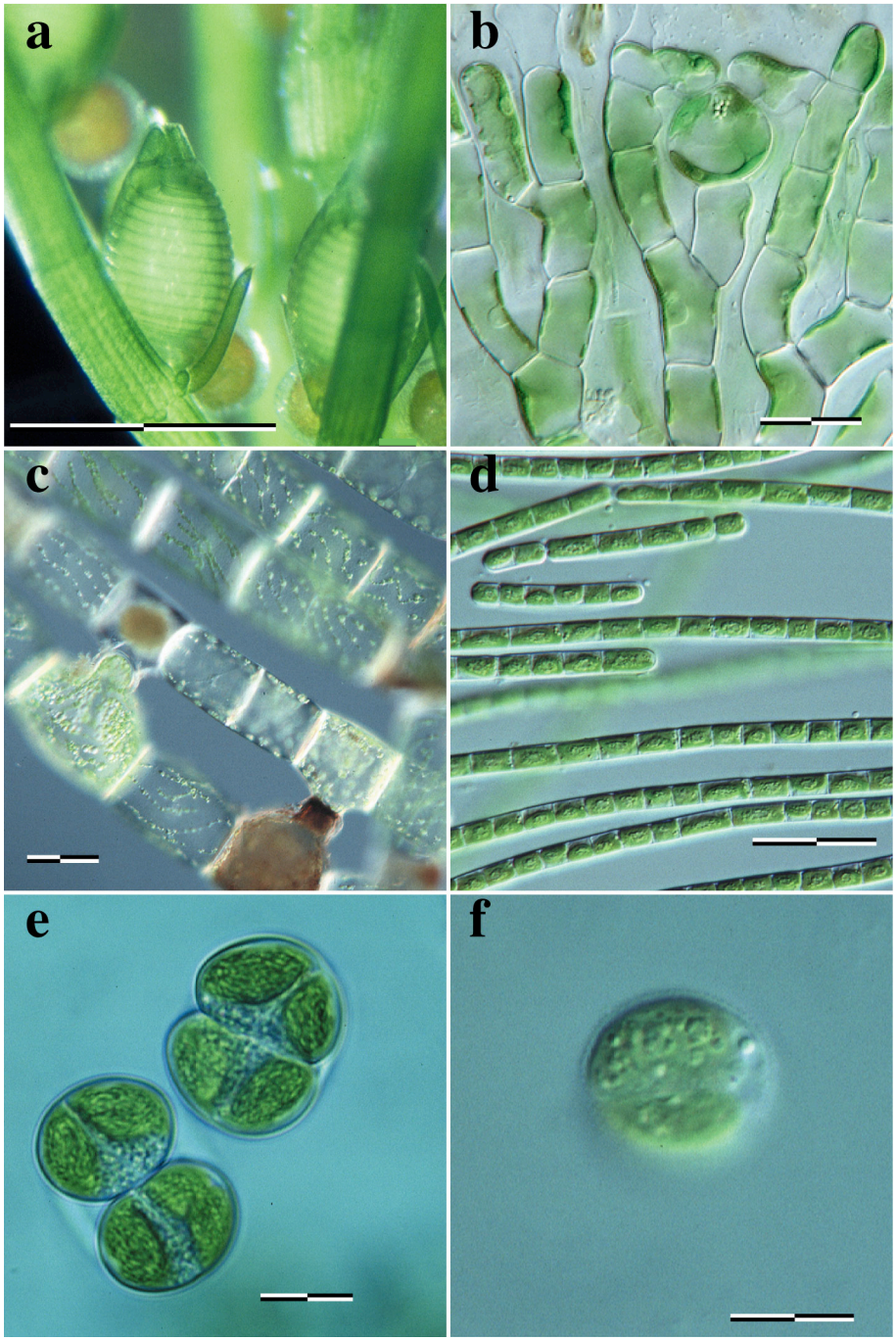
In this dissertation, the evolutionary history of the Charales was investigated by means of several interrelated phylogenetic analyses. First, a multi-gene phylogenetic analysis is presented to address the phylogenetic position of the Charales in the larger scheme of green plant evolution. Second, data derived from the Charales fossil record are combined with DNA sequence data to estimate the absolute divergence times of the extant members of the Charophyta. Finally, a phylogenetic framework for the genus *Nitella* is presented. This study includes a broad representative of species from around the world and serves as a starting point for testing the classification scheme of Wood (1965).

Chapter II: The closest living relatives of land plants

Introduction

The evolutionary origin of the land plants (or embryophytes) from their green algal ancestor was a pivotal event in the history of life. This monophyletic group, including bryophytes, pteridophytes, and seed plants, has altered the biosphere and now dominates the terrestrial environment, but uncertainty as to the identity of their closest living relatives persists in the literature after more than a century of scrutiny (Bower, 1908; Graham, 1993; Kenrick and Crane, 1997a). Morphological and molecular studies have identified two distinct lineages within the green plants *sensu lato*, termed Charophyta and Chlorophyta. The Charophyta comprise the land plants and at least five lineages (orders) of fresh water green algae (Figure II-1) the latter recognized as the class Charophyceae (Mattox and Stewart, 1984; Pickett-Heaps and Marchant, 1972). The Charophyta are sister to the Chlorophyta, which consist of essentially all other green algae. Previous molecular analyses have verified monophyly of most of the charophyte orders (Chapman et al., 1998; Gontcharov et al., 2003; Marin and Melkonian, 1999; McCourt et al., 2000), but branching patterns among these lineages have been only weakly supported, with results that were sensitive to taxon selection and method of phylogenetic reconstruction (McCourt, 1995). Similarly, analyses of morphological and genome structural data have clarified some relationships (Graham et al., 1991; Manhart and Palmer, 1990; Mishler et al., 1994; Qiu et al., 1998; Sluiman, 1985), but have been limited by the number of available characters, number of genomes sequenced, uncertain homology assessment, and a lack of character independence.

Figure II-1. Representative green algal species in the Charophyta. **(1a)**, *Chara globularis* (Charales) KGK0044, showing cortication of developing oogonium and antheridium in the background. Freshwater, scale bar 1 mm. **(1b)**, *Coleochaete pulvinata* (Coleochaetales) CFD 56a6, showing early developmental stage of zygote cortication. Freshwater, scale bar 30 μm. **(1c)**, *Spirogyra maxima* (Zygnematales) UTEX 2495, showing conjugation tubes and partially developed zygotes. Freshwater, scale bar 100 μm. **(1d)**, *Klebsormidium nitens* (Klebsormidiales) SAG 335-2b, showing a single parietal chloroplast per cell. Moist soils or freshwater, scale bar 30 μm. **(1e)**, *Chlorokybus atmosphyticus* (Chlorokybales) UTEX 2591 growing in characteristic sarcinoid packets of cells. Moist soils, scale bar 10 μm. **(1f)**, *Mesostigma viride* (Mesostigmatales) SAG 50-1. Note surface scales visible on upper portion of cell; flagella are not visible, but would emerge from the medial groove in direction of viewer. Freshwater, scale bars 10 μm. Micrographs 1b and 1c-1f kindly provided by C. F. Delwiche and 1c by C. S. Drummond.


a
b
c
d
e
f

It is not surprising that identifying the closest living relatives of land plants has been difficult. Roughly 470 million years of evolution since the colonization of the land, coupled with rapid radiation and numerous extinction events (Feist and Grambast-Fessard, 1991; Graham, 1993; Grambast, 1974; Kenrick and Crane, 1997a; Peck, 1953), has resulted in an inherently difficult phylogenetic problem. Under such conditions, homology assessment can be difficult and phylogenetic reconstruction complex (Felsenstein, 1978). Other factors complicating this phylogenetic problem are the relatively weak signal from any single gene, and unrealistic assumptions made by some analytical methods. It is possible to compensate in part for these difficulties with thorough taxon sampling (Rannala et al., 1998) (to the extent possible given extinction), combined with a relatively large number of characters from concatenated genes (Graham and Olmstead, 2000; Lemieux et al., 2000; Pryer et al., 2001; Qiu et al., 1999; Soltis et al., 1999; Turmel et al., 2002b) and methods of phylogenetic reconstruction that incorporate biologically meaningful models (Hillis et al., 1994). Here a molecular phylogenetic analysis is presented using DNA sequence data from four genes representing three plant genomes: *atpB* and *rbcL* (plastid), *nad5* (mitochondrial), and SSU rRNA gene sequences (nuclear) and the implications of this phylogeny are considered to address the evolutionary origin of land plants and to identify the closest living relatives of this group.

Materials and Methods

DNA isolation, PCR, and sequencing

Total cellular DNA was isolated by the CTAB method (Doyle and Doyle, 1987), UNSET method (a high-urea, SDS extraction buffer) or using the Nucleon Phytopure resin-based extraction kit following the protocol provided for small samples (Amersham Pharmacia, Uppsala, Sweden). Thalli were acquired either from cultures growing in uni-algal condition or from nature. Contaminating epiphytes were removed from natural collections and cleaned thalli were placed at –20º C until time of extraction. In total, thirty-four representative charophytes were sampled, including eight land plants, as well as six outgroup taxa. A summary of species, source, and GenBank accession numbers is shown in Table II-1.

TABLE II-1. Lists of species, source, and GenBank accession numbers. The column labeled 'nrSSU Δ total/unamb.' shows total number of conflicting characters (unambiguous, ambiguous, and inferred indels) compared to published sequences, and total differences comparing only unambiguous characters between new and published sequences. All comparisons reflect sequences determined from the identical algal strain or DNA extraction as the published sequence. 'na' = not applicable.

	Strain	*atpB*	*rbcL*	*nad5*	nrSSU	nrSSU Δ total/unamb.
Land plants						
Arabidopsis thaliana		AP000423	AP000423	NC001284	AC006837	na
Taxus spp.		AJ235619	AJ235811	AJ000705	D16445	na
Psilotum nudum		U93822	U30835	AJ012794	U18519	na
Dicksonia antarctica		U93829	U05919	AJ130745	U18624	na
Huperzia spp.		U93819	X98282	AJ012795	U1850	na
Sphagnum spp.		AF313557	L13485	AJ001225	Y11370	na
Anthoceros/*Phaeoceros* spp.		D86545	D43696	AJ000698	U18491	na
Marchantia polymorpha		X04465	X04465	NC001660	AB021684	na
Charales						
Chara connivens	F140	AF408782	AF097161	AF408200	AF408223	na
Lamprothamnium macropogon	X695	AF408783	U27534	AF408201	AF408224	16/6
Lychnothamnus barbatus	159	AF408784	AF097171	AF408202	AF408225	na
Nitellopsis obtusa	F131B	AF408785	U27530	AF408203	AF408226	na
Nitella opaca	F146	AF408786	AF097174	AF408204	AF408227	na
Tolypella prolifera	F150	AF408787	AF097175	AF408205	AF408228	na
Coleochaetales						
Coleochaete orbicularis	UTEX LB 2651	AF408788	L13477	AF408206	AF408229	28/14
Coleochaete soluta	CFD 32d1	AF408789	AF408247	AF408207	AF408230	na
Coleochaete irregularis	CFD 3d2	AF408790	AF408248	AF408208	AF408231	na
Coleochaete sieminskiana	CFD 10d1	AF408791	AF408249	AF408209	AF408232	na
Chaetosphaeridium globosum	SAG 26.98	AF408792	AF408250	AF408210	AF113506[4]	na
Chaetosphaeridium ovalis	CFD 5c1	AF408793	AF408251	AF408211	AF181094	na
Zygnematales						
Zygnema peliosporum	UTEX LB 45	AF408799	U38701	AF408215	AF408238	na
Spirogyra maxima	UTEX LB 2495	AF408797	L11057	nd	AF408236	na
Mougeotia sp.	UTEX LB 758	AF408800	AF408252	AF408216	AF408239	134/>50
Mesotaenium caldariorum	UTEX 41	AF408798	U38696	nd	AF408237	10/2
Gonatozygon monotaenium	UTEX LB 1253	AF408796	U71438	AF408214	AF408235	na
Onychonema sp	UTEX LB 832	AF408794	AF203501	AF408212	AF408233	na
Cosmocladium perissum	UTEX LB 2447	AF408795	AF203494	AF408213	AF408234	na
Klebsormidiales						
Klebsormidium flaccidum	UTEX LB 2017	AF408801	L13478	AF408217	AF408240	6/0
Klebsormidium subtilissimum	UTEX 462	AF408802	AF408253	AF408218	AF408241	na
Klebsormidium nitens	SAG 335-2b	AF408803	AF408254	AF408219	AF408242	1/0
Entransia fimbriata	UTEX LB 2353	AF408804	AF203496	AF408220	AF408243	na
Chlorokybales						
Chlorokybus atmosphyticus	UTEX LB 2591	AF408805	AF408255	AF408221	AF408244	14/3
Mesostigmatales						
Mesostigma viride	SAG 50-1	AF408806	AF408256	AF408222	AF408245	3/2
Mesostigma viride		AF166114	AF166114	AF353999	AJ250109	na

	Strain	*atpB*	*rbcL*	*nad5*	nrSSU	nrSSU Δ total/unamb.
Outgroups						
Volvox carteri		AB013999	D63446	nd	X53904	na
Chlamydomonas spp.		M13704	J01399	U03843	U70786	na
Paulschulzia pseudovolvox	UTEX 167	AB014040	D86837	nd	AF408246	na
Pteromonas spp.		AB014038	AJ001887	nd	X91627	0/0
Nephroselmis olivacea		NC000927	NC000927	NC000892	X74754	na
Cyanophora paradoxa		U30821	U30821	unpublished	X68483	na

The polymerase chain reaction (PCR) with gene specific primers was used to amplify each of the four genes. The resulting PCR products were purified using a polyethylene glycol (PEG) precipitation (Morgan and Soltis, 1993). Sequences were determined on both strands with the PCR primers and internal sequencing primers. Several particularly divergent sequences required sequence-specific internal primers (Table II-2). Sequencing reactions were performed with the BigDye Terminator Ready Reaction Kit v2.0 according to the manufacturer's protocol (Perkin Elmer Biosystems, Foster City, CA) and resolved using either an ABI-PRISM 377 or 3100 DNA sequencer. The resulting sequence chromatograms were edited and compiled into a single alignment using Sequencher 3.1.1 (Gene Codes Corporation, Ann Arbor, MI) and exported in NEXUS format for phylogenetic analyses. Many previously published SSU rRNA gene sequences were difficult to align to putative secondary structure models and were re-sequenced for this study (Table II-1). For re-sequenced individuals the SSU rRNA gene was amplified on two ways, (1) with PCR primers used to amplify the original sequence Table II-2), and (2) with primers used for all new SSU sequences in this study. Each amplicon was sequenced separately and then compared. With this procedure, the possibility of primer-specific amplification could be eliminated. Careful attention was made to re-sequence the SSU rRNA genes from either identical strain material or the DNA extraction used to generate the original sequence.

Table II-2. PCR and sequencing primers used in this study. A single asterisks '*' indicates primers used for both PCR and sequencing. A double asterisk '**' denotes primers used only for sequencing.

Gene	Primer sequence 5'-3'	Taxon Range
atpB		
175 Forward*	TGTTACTTGTGAAGTTCAACA	Universal
1404 Reverse*	CTAAATAAAATGCTTGTTCAGG	Universal
835F**	GCTGGTTCGGAAGTTTCTGC	Universal
722R**	GGAGGCTCATTCATTTGACC	Universal
865F**	TACTGTGCTTTCTCGTAATC	Characeae
901R**	ATAGTAGAAGTAGAATCAAG	Characeae
835F**	GCAGGATCCGAGGTGTCTGC	*Mougeotia*
722R**	GGTGGTTCGTTCATCTGACC	*Mougeotia*
rbcL		
RH1 Forward*	ATGTCACCACAAACAGAAACTAAAGC	Universal
1385 Reverse*	AATTCAAATTTAATTTCTTTCC	Universal
715F**	TATCTGAATGCTACTGCTGG	Universal
734R**	CCAGCAGTAGCATTCAGATA	Universal
670F**	GCAATTTATAAATCTCAAGCAG	Characeae
751R**	GCATTTCTTCACAAGTTCCTG	Characeae
nad5		
nad5 Forward*	GTAGGTGATTTTGGATTAGC	Universal
nad5 Reverse*	GTACCTAAACCAATCATCATATC	Universal
946F**	ACTTGTAGTCAATTAGGTTATATG	Universal
1109R**	CCCATTTTTCGCATATCTTGTTCATC	Universal
Klebnad5F**	ATGAACCATGCTTTTTTTAAAGC	*Klebsormidium*
Klebnad5R**	GACATTGCATGAATTACAGACCCAG	*Klebsormidium*
Entnad5R**	GCATGAATAACGGATCCGGCAC	*Entransia*
SSU		
Medlin-5'	AACCTGGTTGATCCTGCCAGT	Universal
Medlin-3'	TGATCCTTCTGCAGGTTCACCTAC	Universal
Wilcox-5'	CTGGTTGATCCTGCCAGTAG	Universal
Wilcox-3'	TGATCCTTCTGCAGGTTCAC	Universal
NS1*	GTAGTCATATGCTTGTCTC	Universal
C-nc18S10*	CTTGTTACGACTTCTCCT	Universal
N-nc18S4**	TGCAGTTAAAAAGCTCGT	Universal
C-nc18S4**	ACGAGCTTTTTAACTGCA	Universal
N18I**	AATTTGACTCAACACGGG	Universal
C18I**	CCCGTGTTGAGTCAAATT	Universal

A single intron was found in the *Coleochaete orbicularis nad5* sequence that was not found in any other charophyte green algal sequence. Introns with the same insertion point were previously identified in *nad5* sequences from the liverwort *Marchantia* and the moss *Sphagnum* (Beckert et al., 1999). An intron of similar size, but different insertion point, was also found in *Anthoceros* (a hornwort). Homology of these introns was assessed by calculating pair-wise similarity values using the program 'GAP' found in the GCG computer package of programs (GCG, 2000). For comparison, random sequences with similar base composition and length to each natural sequence were generated with MacClade v4.01 (Maddison and Maddison, 2001) and also analyzed using GAP.

Phylogenetic analyses

Bayesian inference (BI) (Huelsenbeck and Bollback, 2001; Huelsenbeck et al., 2001; Larget and Simon, 1999; Rannala and Yang, 1996) was carried out using MrBayes v2.0 (Huelsenbeck and Ronquist, 2001). MrBayes uses a Metropolis-coupled Markov chain Monte Carlo (or MCMCMC) algorithm that runs several chains simultaneously. Two separate runs were carried out with four Markov chains, each starting from a random tree. Three of these chains were heated allowing for broad sampling of parameter space while the forth chain was not. The Markov chains were run for two million generations sampling every 100 generations for a total of 20,000 samples each run. The first 1,000 samples from each run were discarded as burn-in (data points sampled before the chain reaches stationarity), and the remaining 38,000 samples (19,000 from each run) were combined into a single file and analyzed using the 'sumt' command

in MrBayes. Both independent runs found essentially identical tree topologies and posterior probabilities (not shown), indicating that the sample number was sufficient to permit the algorithm to converge on a global solution.

Maximum likelihood (ML) analyses were carried out using PAUP* v4.0b8 (Swofford, 1998). Using the likelihood ratio test statistic, model selection procedures (Goldman, 1993) identified the general-time-reversible model (Yang, 1994a) with invariable sites (Hasegawa et al., 1985) and gamma-distributed rates (Yang, 1994b) for variable sites (GTR+I+Γ) as the best-fitting model. With model parameters estimated from the data, a heuristic search with ten random taxon addition sequences, TBR branch swapping, and steepest descent option active was performed. Bootstrap analyses were conducted using 500 resampling replicates generated by CodonBootstrap v3.0b4 (Bollback, 2001) taking into account protein-coding and ribosomal portions of the dataset. For each bootstrap replicate, a heuristic search with three random taxon addition sequences, NNI branch swapping, and steepest descent option active was performed.

Analyses using maximum parsimony (MP), and minimum evolution (ME) with two distance measures (LogDet [ME-ld] and maximum likelihood [GTR+I+Γ; ME-ml] distances) were also performed using PAUP*. For both MP and ME analyses, a heuristic search was performed with ten random taxon addition replicates, TBR branch swapping, and steepest descent option active. Bootstrap analyses were conducted using 1,000 resampling replicates.

The Kishino-Hasegawa (KH) test (Kishino and Hasegawa, 1989) was used to evaluate differences between the best phylogeny with those corresponding to alternate hypotheses. Two alternative hypotheses of the sister taxon to the land plants were

evaluated: (1) Coleochaetales sister to land plants, and (2) Charales sister to Coleochaetales and these sister to land plants. Bayesian posterior probabilities and bootstrap analyses permit evaluation of alternative hypotheses and both were used to evaluate these alternative hypotheses.

Results

Taxa and data set

The data set used for phylogenetic analyses excludes introns and unalignable regions for a total length of 5,147 base pairs. Unalignable regions included a forty-five-character region of *nad5* and a five, three, and six character region of the SSU gene, all of which corresponded to putative loop regions. Mean base composition across taxa and number of parsimony informative characters for each gene and the combined data set are presented in Table II-3.

Table II-3. Mean base composition values across taxa, parsimony informative characters (PIC), parsimony uninformative characters (PUC), constant characters (CC), and total characters (Total) for *atpB*, *rbcL*, *nad5*, SSU and all four genes combined.

	%A	%C	%G	%T	PIC	PUC	CC	Total
atpB	31	17	21	31	555	62	589	1,206
rbcL	28	18	23	31	593	83	677	1,353
nad5	24	19	20	37	443	123	175	741
SSU	25	21	28	26	329	211	1,307	1,847
All data	27	19	23	31	1920	479	2,748	5,147

A single intron was identified in the *Coleochaete orbicularis nad5* sequence that was not found in any other species of *Coleochaete* or any other algal charophyte *nad5* sequence sampled. Introns with the same insertion point as that of *C. orbicularis* were previously identified in *Sphagnum* and *Marchantia* (Beckert et al., 1999) and sequence identity scores (Table II-4) generated using 'GAP' revealed of 69.39% identity between *Sphagnum* and *Marchantia*. These sequences compared with *C. orbicularis* shared only 37.82% and 37.81%, respectively. *Anthoceros* has an apparently unrelated intron inserted 128 base pairs downstream with 37.35% identity with that of *Sphagnum*, 35.99% identity to *Marchantia*, and 39.46% to *C. orbicularis*. For comparison, pairs of random sequences with similar base composition and length as the natural sequences had an average of 37.78% sequence identity. These data not only confirm the independent origin of the intron found in *Anthoceros* but also suggest that the *C. orbicularis nad5* intron was acquired independently from that shared by *Sphagnum* and *Marchantia*.

The best fitting likelihood model of sequence evolution as determined with the likelihood ratio test was the general-time-reversible model with invariable sites and a gamma distributed correction for rate variation among variable sites (GTR+I+Γ). The parameter estimation procedure converged on identical values after two iterations and these values were fixed for both ML and ME-ml analyses. ME-ld analyses used the same fraction of invariant sites (I=0.388199) estimated my ML. In the following discussion, PP = Bayesian posterior probability; BS = bootstrap support.

TABLE II-4. Pair-wise comparisons of *nad5* intron sequences. Percent similarity between *Coleochaete orbicularis* (1), *Marchantia*, *Anthoceros*, *Sphagnum*, and four random sequences of similar base composition and length as the natural sequences (*).

	Coleochaete[1]	*Marchantia*	*Anthoceros*	*Sphagnum*	*Coleochaete**	*Marchantia**	*Anthoceros**	*Sphagnum**
Coleochaete[1]	-	37.828	36.617	37.823	37.072	42.471	35.120	38.122
Marchantia		-	35.928	69.360	36.364	39.841	36.677	38.623
Anthoceros			-	37.349	36.969	36.963	36.988	36.461
Sphagnum				-	40.111	37.164	36.574	36.091
*Coleochaete**					-	40.602	39.556	41.155
*Marchantia**						-	40.149	40.625
*Anthoceros**							-	36.232
*Sphagnum**								-

Using BI and ML on the combined four-gene data set (Figure II-2), the order Charales was found sister to the land plants with strong statistical support (PP=1.0, BS=94) and a monophyletic Coleochaetales sister to the Charales/land plant clade (PP=1.0, BS=59). The MP and ME analyses (Figure II-3) also support the result that Charales have a closer relationship to land plants than do Coleochaetales (MP=80, ME-ld=97, ME-ml=92). The overall structure of the best tree is consistent with previous work in that the classically recognized orders were also recovered (land plants, PP=1.0, ML=100, MP=100, ME-ld=100, ME-ml=100; Charales, PP=1.0, ML=100, MP=100, ME-ld=100, ME-ml=100; Coleochaetales, PP=1.0, ML=62, MP=<50, ME-ld=75, ME-ml=<50; Zygnematales, PP=1.0, ML=99, MP=93, ME-ld=68, ME-ml=<50; and Klebsormidiales PP=1.0, ML=100, MP=100, ME-ld=100, ME-ml=100). There was also support for placement of the enigmatic filamentous alga *Entransia* (McCourt et al., 2000) with the Klebsormidiales (PP=1.0, ML=77, MP=77, ME-ld=<50, ME-ml=64). The rare, monotypic genus *Chlorokybus* was found sister to the remainder of the unambiguous charophytes, while all analyses strongly support the inclusion of *Mesostigma* within the Charophyta (PP=1.0, ML=97, MP=100, ME-ld=100, ME-ml=100).

FIGURE II-2. Phylogenetic relationships for the Charophyta determined by Bayesian inference from the combined four-gene data set. The maximum likelihood tree (-ln = 64499.87863) was of identical topology. Posterior probabilities are noted above branches and maximum likelihood bootstrap values are below branches. The topology is drawn with *Cyanophora* rooting the tree. Branch lengths are Bayesian mean values and are proportional to the number of substitutions per site (scale bar, 0.05 substitutions/site). Taxonomy is modified from Mattox and Stewart (1984).

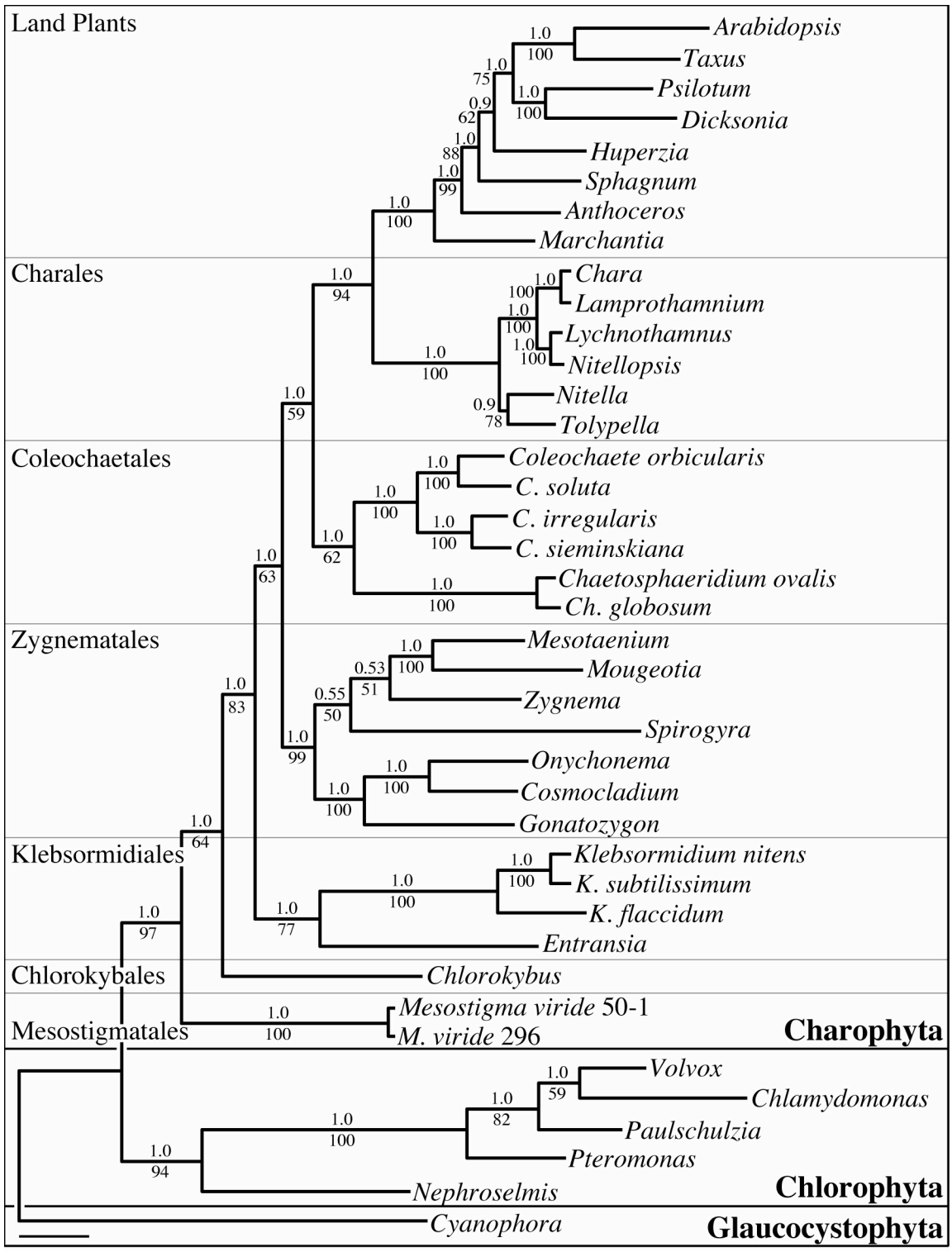
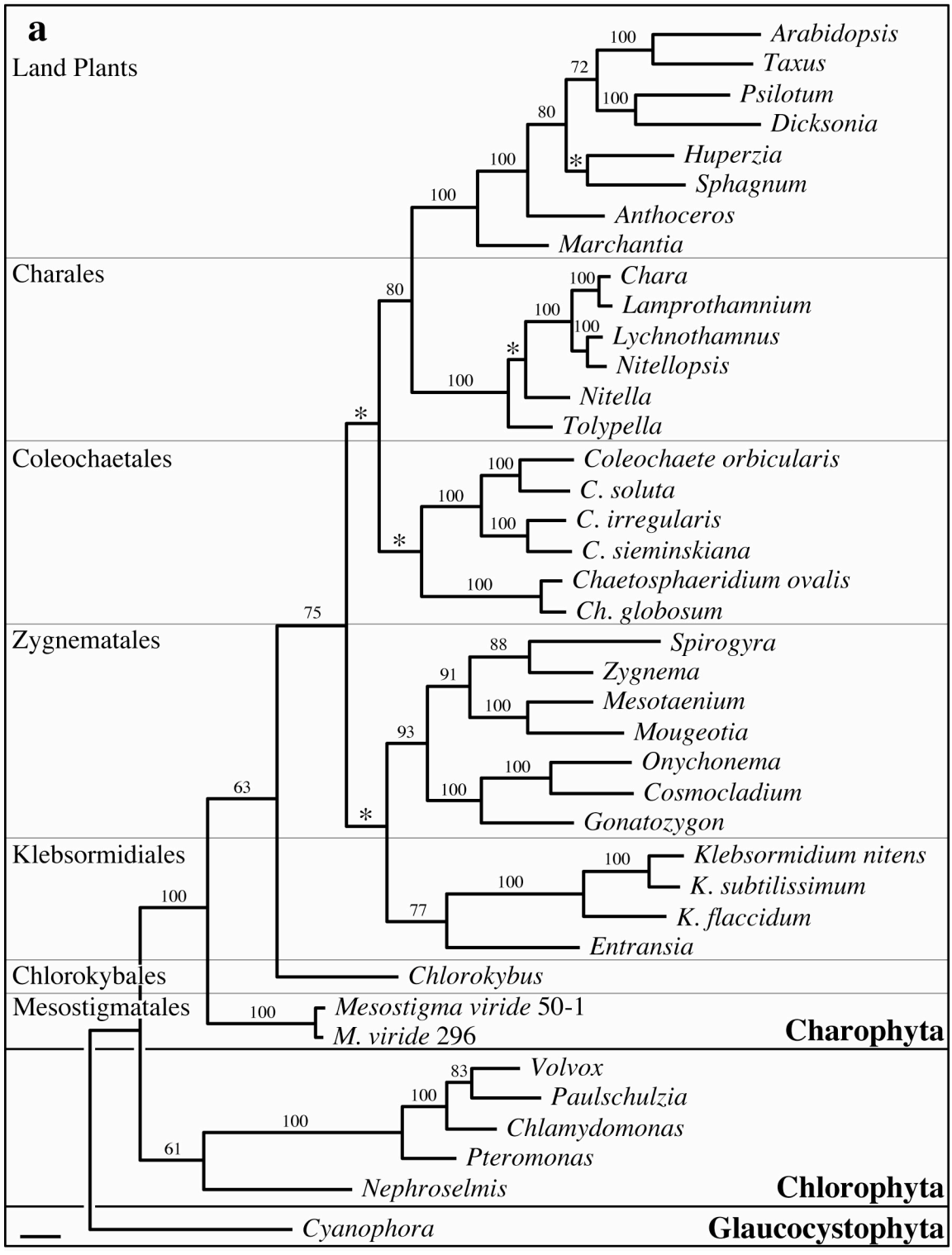
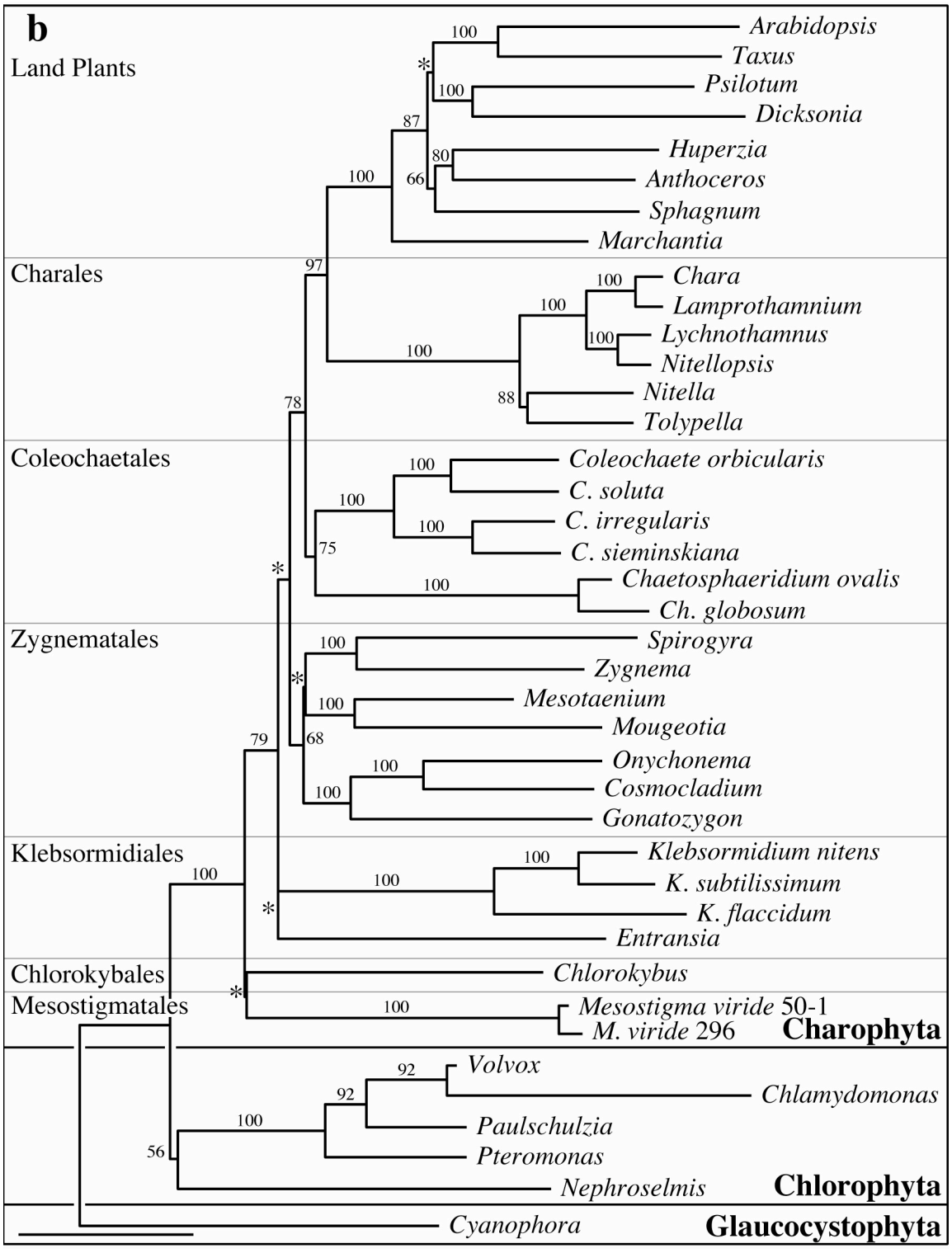
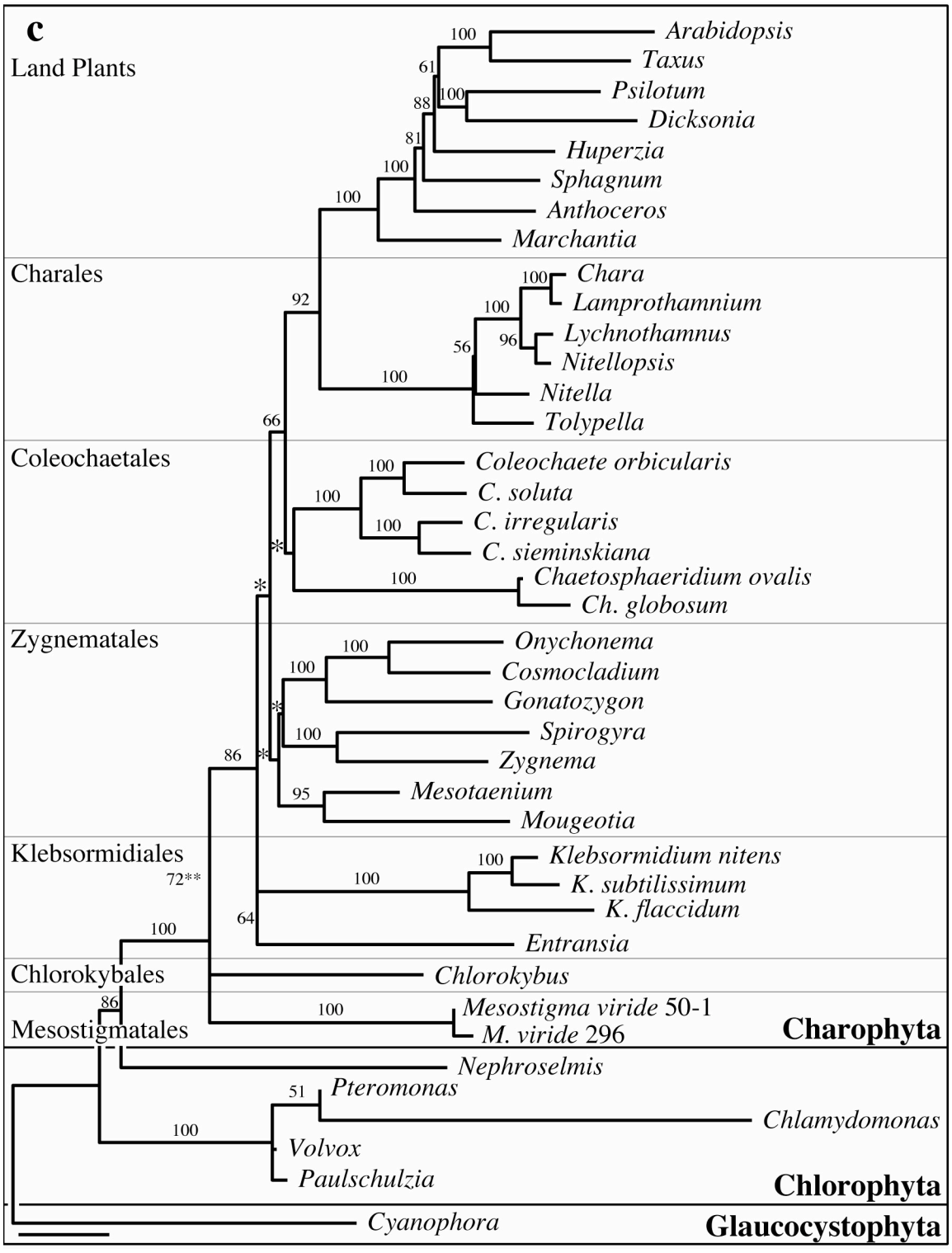

Land Plants
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Charales
Chara
Lamprothamnium
Lychnothamnus
Nitellopsis
Nitella
Tolypella
Coleochaetales
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum
Zygnematales
Mesotaenium
Mougeotia
Zygnema
Spirogyra
Onychonema
Cosmocladium
Gonatozygon
Klebsormidiales
Klebsormidium nitens
K. subtilissimum
K. flaccidum
Entransia
Chlorokybales
Chlorokybus
Mesostigmatales
Mesostigma viride 50-1
M. viride 296
Charophyta
Volvox
Chlamydomonas
Paulschulzia
Pteromonas
Nephroselmis
Chlorophyta
Cyanophora
Glaucocystophyta

FIGURE II-3. Phylogenetic relationships of the Charophyta inferred from the combined four-gene data set using additional analytical methods. Bootstrap values (1000 replicates) are shown above branches. An asterisk '*' denotes bootstrap values below 50%. **(a)** Maximum parsimony tree (tree score, 12,365 steps; scale bar, 100 substitutions). **(b)** Minimum evolution tree using LogDet distances (tree score, 2.50594; scale bar, 0.05 substitutions/site). **(c)** Minimum evolution tree using maximum likelihood distances (tree score, 3.57107; scale bar, 0.05 substitutions/site). ** Bootstrap value for a very short branch uniting *Chlorokybus* with the remaining traditional charophyte lineages excluding *Mesostigma*. The topologies are drawn with *Cyanophora* rooting the trees. Taxonomy is modified from Mattox and Stewart (1984).

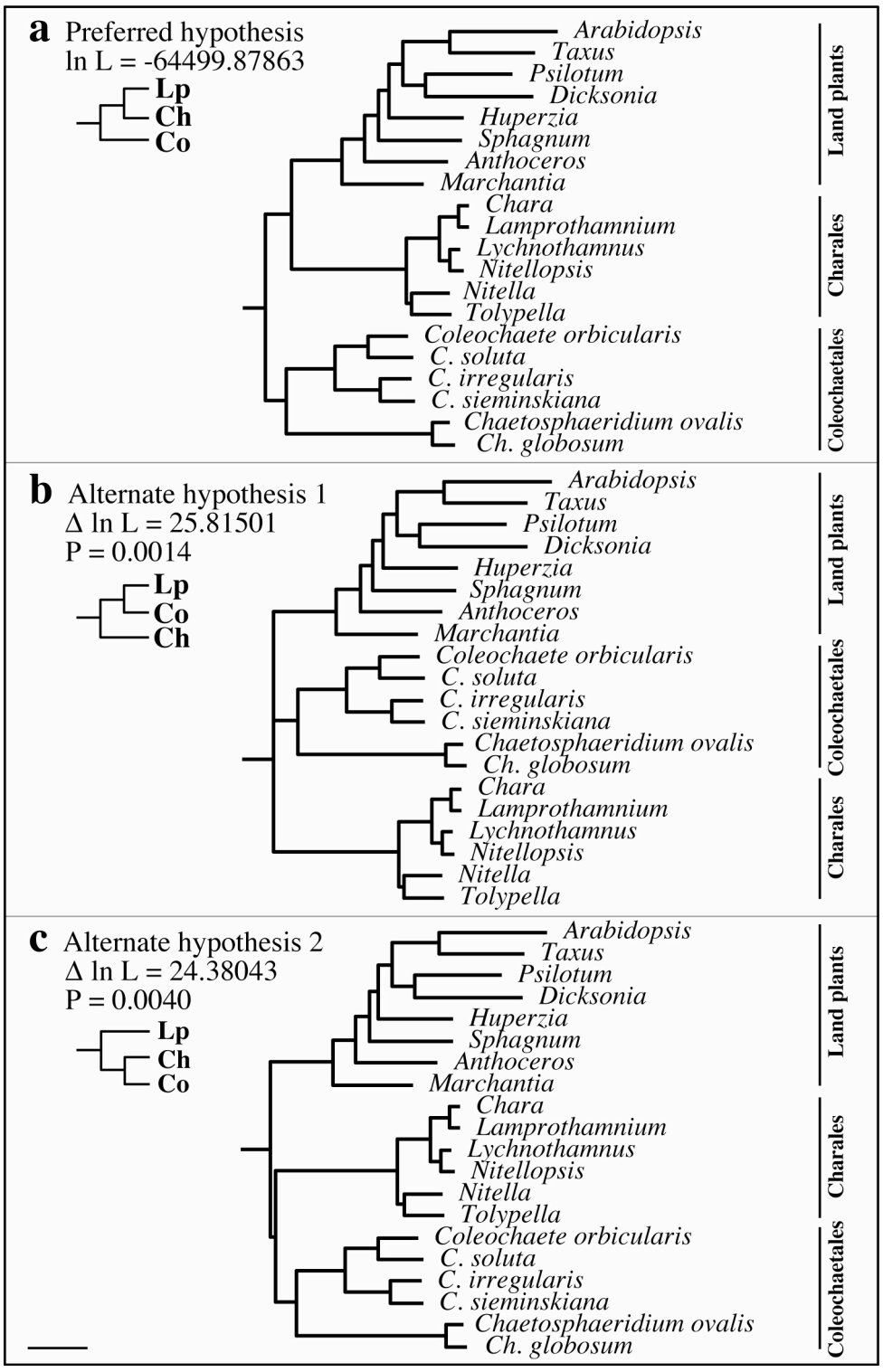

a
Land Plants
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Charales
Chara
Lamprothamnium
Lychnothamnus
Nitellopsis
Nitella
Tolypella
Coleochaetales
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum
Zygnematales
Spirogyra
Zygnema
Mesotaenium
Mougeotia
Onychonema
Cosmocladium
Gonatozygon
Klebsormidiales
Klebsormidium nitens
K. subtilissimum
K. flaccidum
Entransia
Chlorokybales
Chlorokybus
Mesostigmatales
Mesostigma viride 50-1
M. viride 296
Charophyta
Volvox
Paulschulzia
Chlamydomonas
Pteromonas
Nephroselmis
Chlorophyta
Cyanophora
Glaucocystophyta


b
Land Plants
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Anthoceros
Sphagnum
Marchantia
Charales
Chara
Lamprothamnium
Lychnothamnus
Nitellopsis
Nitella
Tolypella
Coleochaetales
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum
Zygnematales
Spirogyra
Zygnema
Mesotaenium
Mougeotia
Onychonema
Cosmocladium
Gonatozygon
Klebsormidiales
Klebsormidium nitens
K. subtilissimum
K. flaccidum
Entransia
Chlorokybales
Chlorokybus
Mesostigmatales
Mesostigma viride 50-1
M. viride 296
Charophyta
Volvox
Chlamydomonas
Paulschulzia
Pteromonas
Nephroselmis
Chlorophyta
Cyanophora
Glaucocystophyta


c
Land Plants
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Charales
Chara
Lamprothamnium
Lychnothamnus
Nitellopsis
Nitella
Tolypella
Coleochaetales
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum
Zygnematales
Onychonema
Cosmocladium
Gonatozygon
Spirogyra
Zygnema
Mesotaenium
Mougeotia
Klebsormidiales
Klebsormidium nitens
K. subtilissimum
K. flaccidum
Entransia
Chlorokybales
Chlorokybus
Mesostigmatales
Mesostigma viride 50-1
M. viride 296
Charophyta
Nephroselmis
Pteromonas
Chlamydomonas
Volvox
Paulschulzia
Chlorophyta
Cyanophora
Glaucocystophyta

Results from the KH test rejected both the Coleochaetales sister to land plants (P=0.0014) and the Coleochaetales sister to Charales (P=0.0040) in favor of Charales sister to land plants (Figure II-4). Both Bayesian posterior probabilities and bootstrap analyses were unable to identify either of these alternative hypotheses with non-trivial support: Coleochaetales sister to land plants, PP=0.0, BS=0.0%; Coleochaetales sister to Charales, PP=0.0, BS=0.4%. Bayesian posterior probabilities and bootstrap analyses were also examined for *Coleochaete orbicularis* sister to land plants, PP=0.0, BS=0.0%; and *Coleochaete* sister to land plants, PP=0.0, BS=0.0%. Taken together, these results strongly support the placement of the Charales sister to the land plant and reject the above-mentioned alternative hypotheses.

FIGURE II-4. Kishino-Hasegawa test results for alternate hypotheses of the sister taxon to land plants. Inset trees diagram alternative hypotheses for relationships among land plants (LP), Charales (Ch) and Coleochaetales (Co). **(a)** Preferred hypothesis generated by both Bayesian inference and maximum likelihood analyses. The log likelihood (ln L) was determined with PAUP* using the GTR+I+Γ model of sequence evolution. **(b)** Alternate hypothesis 1 showing the maximum likelihood topology constraining Coleochaetales to be sister to land plants. The Δ ln L value indicates the difference in log likelihood relative to the preferred hypothesis and the P value represents the probability of getting a more extreme t-value under the null hypothesis of no difference between the two hypotheses (significant at $P < 0.05$, one-tailed test). **(c)** Alternate hypothesis 2 constraining Charales and Coleochaetales to be monophyletic and sister to land plants. Scale bar is equal to 0.05 substitutions/site.


a
Preferred hypothesis
ln L = -64499.87863
Lp
Ch
Co
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Chara
Lamprothamnium
Lychnothamnus
Nitellopsis
Nitella
Tolypella
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum
Land plants
Charales
Coleochaetales
b
Alternate hypothesis 1
Δ ln L = 25.81501
P = 0.0014
Lp
Co
Ch
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum
Chara
Lamprothamnium
Lychnothamnus
Nitellopsis
Nitella
Tolypella
Land plants
Coleochaetales
Charales
c
Alternate hypothesis 2
Δ ln L = 24.38043
P = 0.0040
Lp
Ch
Co
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Chara
Lamprothamnium
Lychnothamnus
Nitellopsis
Nitella
Tolypella
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum
Land plants
Charales
Coleochaetales

Discussion

Both Charales and Coleochaetales have long been considered to be close relatives of the land plants (Bower, 1908; Fritsch, 1935; Graham, 1993; Kenrick and Crane, 1997a; Mattox and Stewart, 1984; Pickett-Heaps and Marchant, 1972). Key morphological characters uniting these three lineages include branched filamentous growth, oogamous sexual reproduction, and phragmoplastic cell division, along with a suite of ultrastructural and biochemical features (Graham and Wilcox, 2000). In light of similar morphological traits (i.e., parenchyma-like tissue, placental transfer cell wall ingrowths and zygote retention), the genus *Coleochaete* and, in some instances, a single species, *C. orbicularis*, has been discussed as a possible sister taxon to land plants (Graham et al., 1991; Mishler et al., 1994). The results here indicate that the Coleochaetales are monophyletic and less closely related to the land plants than the Charales.

The Charales also share numerous characteristics with land plants, some of which are not found in the Coleochaetales. These include gross sperm morphology and ultrastructure (Duncan et al., 1997; Steil, 1941), numerous discoidal chloroplasts per cell, protonemal filaments, complete absence of zoospores (sperm are the only flagellate cells), and encasement of the egg by sterile jacket cells (cortication) prior to fertilization (Fritsch, 1935; Graham and Wilcox, 2000). The results of this study suggest that many of the similarities between Charales and land plants may reflect homology rather than convergent evolution. Cortication of the zygote reminiscent of that in Charales is found in some species of *Coleochaete*, but occurs only after fertilization of the egg, and zygote cortication is not thought to occur in *Chaetosphaeridium* (Thompson, 1969). In addition,

primary plasmodesmata have been confirmed in the Charales, a character shared with land plants (Cook et al., 1997). Although plasmodesmata have been described in *Coleochaete* (Stewart et al., 1973), it is unknown whether their development is primary or secondary in nature.

The phylogenetic placement of *Mesostigma*, a unicellular, scaly green flagellate has been controversial and the results presented here shed light on this problem. Traditionally classified with like forms as a prasinophyte, the identification of a land-plant-like MLS led to the suggestion that *Mesostigma* may be more closely allied with the Charophyta than to other scaly flagellates (Melkonian, 1989; Rogers et al., 1981). Like the results presented here, analyses of actin sequences placed *Mesostigma* at the base of the Charophyta (Bhattacharya et al., 1998), and analyses of SSU rRNA gene sequence data place it among them (albeit in close association with *Chaetosphaeridium*, a grouping not supported by other data) (Gontcharov et al., 2003; Marin and Melkonian, 1999; Sluiman and Guihal, 1999). By contrast, maximum likelihood analyses of amino-acid data from both the plastid and mitochondrial genomes of *Mesostigma* find strong support for placement of this genus as sister to all green algae rather than as a basal charophyte lineage (Lemieux et al., 2000; Turmel et al., 2002b). The latter analyses differ from those presented here in the number of characters and taxa sampled (eight vs. forty). When divergence times are large and internal branches short, limited taxon sampling can lead to inaccurate phylogenies (Felsenstein, 1978). If taxon sampling explains this conflict, then one would predict convergence on the phylogeny presented here as additional organellar genomes become available. The phylogenetic position of *Mesostigma* is critical to

understanding the evolution of form and structure in the lineage that gave rise to land plants and warrants further research.

Identification of the Charales as the sister taxon to land plants with the Coleochaetales as sister to the Charales/land plant clade suggests that the common ancestor of land plants was a branched, filamentous organism with a haplontic life cycle and oogamous reproduction. The early stages of development in the Charales involve formation of protonemal filaments reminiscent of those found in some mosses and other land plants, which suggests that a similar heteromorphic development might have occurred in the common ancestor. Other characteristics of this ancestor, including both developmental and biochemical features, may explain not only how their descendants came to survive on land, but also how they ultimately came to dominate terrestrial ecosystems. Moreover, the charophytes have important applications in a wide range of disciplines (Charales in cell biology, Coleochaetales in ultrastructure, and Zygnematales in physiology) (Graham and Wilcox, 2000). Consequently, a robust phylogeny relating these taxa to land plants can place this work in an evolutionary context and lead to the identification and development of appropriate model systems for future studies.

Although it is tempting to envision the origin of land plants as having been from amorphous pond scum, these data indicate that the common ancestor of land plants and their closest algal relatives was a relatively complex organism. The extant Charales are the remnants of a once diverse, but now largely extinct, group which includes some of the oldest known plant fossils (roughly 420 ma, from the late Ordovician) (Feist and Grambast-Fessard, 1991; Grambast, 1974). While the fossil record for the other charophyte orders is fragmentary at best (Tappan, 1980), the molecular phylogenetic data

presented here (Figure II-2, II-3) suggests that these lineages diversified more than 470 ma. While not species rich, these algae hold a key position in the tree of life and, consequently, represent an important part of eukaryotic diversity.

Chapter III: Estimating divergence times in the Charophyta

Introduction

Overview

One of the key events in the history of life was undoubtedly the transition of green algae from their aquatic environment to the land, an event that occurred at least half a billion years ago. This transition and the succeeding diversification not only modified the atmosphere and transformed the physical landscape, but also set the stage for subsequent diversification of numerous other organisms. Botanists, paleontologists and molecular biologists alike have spent considerable energy toward understanding the patterns and processes involved in the evolutionary origin of land plants. Nevertheless, our knowledge of this event has been hampered by two key problems: 1) Circumscription and phylogenetic relationships among extant algal lineages and land plants have been difficult to resolve both with morphology and single-gene data sets (see Chapter II and references therein), and 2) early land plant fossils are inadequate to reconstruct early stages of land plant ancestry (Graham, 1993; Kenrick and Crane, 1997a).

Recent theoretical and technological innovations coupled with improved understanding of green plant diversity have permitted collection and analysis of molecular data (DNA sequences) that more fully represent extant diversity within the green plant lineage Charophyta (Graham and Olmstead, 2000; Lemieux et al., 2000; Pryer et al., 2001; Qiu et al., 1999; Qiu et al., 2001; Soltis et al., 1999; Turmel et al., 2002b). This monophyletic group includes at least six green algal lineages, Charales, Coleochaetales, Zygnematales, Klebsormidiales, Chlorokybales, and Mesostigmatales, as

well as land plants (embryophytes). The four-gene phylogentic analysis discussed in Chapter II presents the first strongly supported evolutionary hypothesis of the early branching events in the Charophyta and identifies the Charales as the sister taxon of land plants. With a robust phylogeny that identifies the branching order among these lineages, it is possible to estimate when the now-extinct ancestors of these lineages diverged and in the process began diversification of what is now the dominant form of terrestrial life.

A common approach, albeit a source of substantial controversy as well, is the use of molecular sequence data to estimate divergence times. Zuckerkandl and Pauling (1962; 1965) were the first to suggest that molecular sequence data might evolve at rates constant enough that molecular divergence measures could be used to calibrate a 'molecular clock.' This assumes that the rate of molecular sequence change is constant across lineages and over time. Under this model, a measurement of the molecular sequence difference between any two lineages would reveal how long ago these lineages diverged from their common ancestor. Numerous studies have used the molecular clock model in attempts to estimate divergence times (Arnason et al., 1998; Cooper and Penny, 1997; Glazko and Nei, 2003; Goremykin et al., 1997; Heckman et al., 2001; Hedges et al., 1996; Wray et al., 1996).

There are, however, considerable problems associated with estimating divergence times under a strict molecular clock (Ayala, 1997; Fitch, 1976; Hillis et al., 1996). A strict clock depends on a uniform substitution rate across lineages and an accurate calibration date for at least one speciation event. However, molecular data sets rarely exhibit constant substitution rates across all taxa (Britten, 1986; Gillespie, 1991; Gissi et al., 2000; Li and Tanimura, 1987; Sanderson and Doyle, 2001). Several methods have

been proposed that attempt to compensate for this. Relative rate tests have been used to identify lineages that significantly deviate from the clock, and once identified, these lineages can be selectively removed before divergence times are estimated (Kooistra and Medlin, 1996; Russo et al., 1995; Takezaki et al., 1995). An obvious drawback to this approach is the loss of data that may be directly pertinent to the question at hand. Another approach invokes 'local clocks' by applying different rate parameters to different parts of the tree (Hasegawa et al., 1989). However, without prior knowledge it might become difficult to identify where or how many local clocks should be placed on a tree. The extreme case would apply separate rate parameters on each branch of the tree; because rate and time are confounded, in this case divergence time estimates would become impossible to calculate. Sanderson (1997) introduced a nonparametric method for smoothing the differences in rates across speciation events on a tree. This method has the advantage of allowing rates to vary on branches while allowing divergence times to be estimated. A similar parametric method has been developed that uses a Bayesian approach for multi-gene data sets (Kishino et al., 2001; Thorne and Kishino, 2002; Thorne et al., 1998). This method, like Sanderson's, assumes that rates are autocorrelated across speciation events; sequences from closely related lineages are assumed to evolve at more similar rates than those from distantly related lineages. Under a Bayesian framework, this method is conditioned on the data and tree topology while integrating over uncertainty in branch lengths, degree of autocorrelation, substitution model parameters, and error associated with fossil calibration dates.

Another important factor that influences the accuracy of divergence time estimates is reliability of the calibration point(s) used for producing the time scale and the

distribution of these calibration points on the phylogenetic tree. Use of calibration points derived from the fossil record assumes that those fossils not only provide an accurate account of the evolutionary history of that lineage but also that the age estimate of the fossil is accurate. Typically, the first occurrence of a taxon in the fossil record is used as a minimum estimate of that lineage's divergence time and should not be interpreted as a maximum age (Doyle and Donoghue, 1993). Upper and lower bounds can be defined to more accurately incorporate the error associated with fossil dates (Sanderson, 1997; Thorne and Kishino, 2002).

The fossil record

Unique among the charophyte algae, a rich and extensively studied fossil record exists for the Charophyceae, here defined as the Charales Lindley and two other orders found exclusively in the fossil state, the Trochiliscales Mädler and the Sycidiales Mädler. Charophyte fossils predominantly consist of the remains of female gametangia (oogonia), called gyrogonites. Oogonia are spherical bodies composed of the single-celled oospore tightly surrounded by a single rank of sterile jacketing cells terminated apically by a set of coronular cells. A basal plate (either one or three sterile sister cells of the oospore), node cell and finally the basal pore subtend the oospore. The oospore itself is invested with a thick layer of sporopollenin, a highly durable material. Complete fossil oogonia are rarely found. Rather, the thickened sporopollenin impregnated oospore membrane with calcified jacket-cell remnants, along with the basal plate impressions, is generally preserved. The orientation of the jacketing cells enclosing the oospore easily differentiates the three orders from one another (Sycidiales, vertical cells; Trochiliscales, dextral cells; Charales, sinistral cells). Fossilized antheridia or vegetative thalli are rarely

seen, but when they are encountered they occur as long internodal cells alternating with short nodal cells bearing whorls of branchlets, which is the arrangement also found in all extant forms (Edwards and Lyon, 1983; Martín-Closas, 1999; Martín-Closas and Diéguez, 1998; Taylor et al., 1992). The presence of sporopollenin in close relatives of the Charophyceae suggests that even the earliest members of this lineage would be capable of fossilization (Delwiche et al., 1989; Graham, 1993; Kroken et al., 1996).

From these fossils, three families are currently recognized in the Sycidiales, two in the Trochiliscales, and six in the Charales (Feist and Grambast-Fessard, 1991). *Praesycidium siluricum* Ishchenko and Ishchenko (Sycidaceae: Sycidiales), from the Late Silurian (Pridolian, 419-417 mya) of Podolia (southwestern Ukraine), is the oldest charophyte species known (Ishchenko and Ishchenko, 1982). *Trochiliscus* (*Eutrochiliscus*) *podolicus* Croft (Trochiliscaceae: Trochiliscales), from the Early Devonian (417-391 mya) of Podolia in Eastern Europe, is the oldest representative of the Trochiliscales (Croft, 1952). Both of these orders persist in the fossil record over relatively short geologic ranges and are not found after the Late Devonian or Early Mississippian (Grambast, 1974).

Within the Charales, six families are recognized and include the only extant family (Characeae). *Eochara wickendeni* Choquette (Eocharaceae), from the Middle Devonian (391-370 mya) of Canada, is the earliest known representative of the Charales and the only known example of this family (Choquette, 1956). *Paleochara acadica* Bell (Paleocharaceae), from the Pennsylvanian (323-290 mya) of Nova Scotia, Canada, is the single representative of this family, being described from only six specimens from the same location (Bell, 1922). The Eocharaceae and Paleocharaceae are unique in the

Charales in that they exhibit more than six and exactly six spiral jacket cells, respectively. Jacket cell number in the remaining families is fixed at five. The Porocharaceae are first seen in the Late Carboniferous (Pennsylvanian, 323-290 mya) and the Clavatoraceae appear in the Late Jurassic (Tithonian, 151-144 mya). Both of these families extend to the Late Cretaceous (Maastrichtian, 71.3-65 mya). At this time, the Raskyellaceae first appear and extend to the Late Oligocene (Chattian, 28.5-23.8 mya). *Aclistochara*, from the Late Triassic, is the oldest representative of the Characeae (Liu and Chen, 1992). This family reached its greatest generic diversity during the Eocene and Oligocene. Diversity then declined rapidly in the Miocene leaving only six extant genera (*Chara*, *Lamprothamnium*, *Nitellopsis*, *Lychnothamnus*, *Nitella*, and *Tolypella*).

It is unclear when the charophyte/land plant ancestor diverged or when the land plants successfully colonized the land. There are no undisputed fossils of charophyte algae before the Silurian or of land plants before the Ordovician (Kenrick and Crane 1997). Because primitive plants and most algae do not preserve well in the fossil record, the possibility exists of an earlier, unrecorded history. Consequently, information derived from the fossil record combined with DNA sequence data are used here to estimate the timing of these events.

Materials and Methods

Taxon sampling

To estimate divergence times in the Charophyta, the 40-taxon data set presented in Chapter II was modified in the following ways, (1) several sequences determined using older manual sequencing methods were re-sequenced with automated methods, (2)

several partial sequences were completed, (3) several sequences were replaced with those from newly published sequences or sequences from the same species, (4) an additional strain of *Entransia fimbriata* (Klebsormidiales) and of *Chlorokybus atmosphyticus* (Chlorokybales) were added to increase sampling of these underrepresented groups, and (5) seven charalean isolates were added to facilitate comparison with the fossil record.

Every effort was made to select charalean taxa that represent not only extant morphological diversity but also molecular diversity within each genus. Preliminary phylogenetic analyses using unpublished *rbcL* sequence data from numerous species of *Chara* and *Lamprothamnium* (R. M. McCourt pers. comm.) were used to determine that *Chara australis* and *C. connivens* together adequately represent the molecular diversity within *Chara*. *Lamprothamnium macropogon* and *L. heraldii* were selected for *Lamprothamnium*. Similarly, the *rbcL* sequence data discussed in Chapter IV were used to select *Nitella hyalina* and *N. opaca* to represent the genus *Nitella*. DNA samples that represent extant diversity across the genus *Tolypella* were not available; however, *Tolypella nidifica* and *Tolypella porteri* were available and used as exemplars for one of the two Sections of *Tolypella* (section Tolypella). Because *Nitellopsis* and *Lychnothamnus* each contain only a single extant species, two geographically disjunct isolates were used for each of these species. Isolates from Germany and China were used for *Nitellopsis obtusa*, and isolates from Croatia and Australia were used for *Lychnothamnus barbatus*. Taxa were chosen to maximize the possibility of reconstructing the deepest (i.e., oldest) branching point for these extant lineages and minimize underestimating divergence times due to inadequate sampling of extant lineages.

DNA isolation, PCR, and sequencing

DNA isolation, PCR, and sequencing of *atpB*, *rbcL*, *nad5* and the small subunit of the nrDNA (SSU) for ten taxa new to this study were performed following the protocols described in Chapter II. Attempts were made to determine *nad5* gene sequences for *Spirogyra* and *Mesotaenium,* both missing *nad5* data in Chapter II. These attempts failed for *Mesotaenium*, but a partial sequence was determined for *Spirogyra*. In summary, new sequences were combined with the modified data set described above for a 50-taxon four-gene data set. A list of species, source, and GenBank accession numbers for new or modified sequences from Table II-1 is shown in Table III-1.

TABLE III-1. Lists of species, source, and GenBank accession numbers for specimens that are either new to this study or modified (in bold face) from Table II-1.

	Strain	*atpB*	*rbcL*	*nad5*	nrSSU
Land plants					
Psilotum nudum		**NC_003386**	**NC_003386**	AJ012794	**X81963**
Dicksonia antarctica		U93829	U05919	AJ130745	**U18624.2**
Huperzia spp.		U93819	X98282	AJ012795	**AF313567**
Anthoceros/*Phaeoceros*		**NC_004543**	**NC_004543**	AJ000698	U18491
Charales					
Chara australis	X067	AY823681	AY823700	AY823690	AY823707
Lamprothamnium heraldii	KGK0069	AY823682	AY823701	AY823691	AY823708
Lychnothamnus barbatus		AF408784	AF097171	AF408202	**AF408225.2**
Lychnothamnus barbatus	3.25.95	AY823683	U27533	AY823692	AY823709
Nitellopsis obtusa	KGK0057e	AY823684	AY823702	AY823693	AY823710
Nitella hyalina	KGK0059b	AY823685	AY823703	AY823694	AY823711
Tolypella nidifica	F138	AY823686	U27531	AY823695	AY823712
Tolypella porteri	X907	AY823687	AY823704	AY823696	AY823713
Coleochaetales					
Chaetosphaeridium globosum		NC_004115	NC_004115	NC_004118	AJ250110
Zygnematales					
Spirogyra maxima	UTEX 2495	AF408797	L11057	AY823697	AF408236
Klebsormidiales					
Klebsormidium flaccidum		**AF408801.2**	L13478	AF408217	AF408240
Klebsormidium subtilissimum		AF408802	**AF408253.2**	AF408218	**AF408241.2**
Klebsormidium nitens		**AF408803.2**	AF408254	AF408219	AF408242
Entransia fimbriata		AF408804	AF203496	AF408220	**AF408243.2**
Entransia fimbriata	MC	AY823688	AY823705	AY823698	AY823714
Chlorokybales					
Chlorokybus atmosphyticus	SAG 34.98	AY823689	AY823706	AY823699	AY823715
Mesostigmatales					
Mesostigma viride		AF408806	AF408256	**AF408222.2**	AF408245
Outgroups					
Chlamydomonas reinhardtii		**BK000554**	**BK000554**	U03843	**M32703**
Pteromonas angulosa		AB014038	AJ001887	nd	**AF395438**
Cyanophora paradoxa	UTEX 555	U30821	U30821	unpublished	AY823716

Phylogenetic analyses

Bayesian inference (BI) was carried out using MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). MrBayes v3.0b4 differs from v2.0 in that the data can be partitioned into subsets of the original data. Implementing this option allows different models to be specified for different data partitions. In Chapter II a single model (GTR+I+Γ) was applied to the entire four-gene data set. A similar analysis was performed here as well as an analysis that partitioned the data by gene. GTR+I+Γ was used for each partition (gene) and the parameters were individually estimated for each partition. For each of these analyses, two separate runs were carried out with four Markov chains, each starting from a random tree. The Markov chains were run for two million generations, sampling every 100 generations, for a total of 20,000 samples per run. The first 1,000 samples from each run were discarded as burn-in (data points sampled before the chain reaches stationarity), and the remaining 38,000 samples (19,000 from each run) were combined into a single file and analyzed using the 'sumt' command in MrBayes. Each independent run with similar model settings found essentially identical tree topologies and posterior probabilities (not shown), indicating that the sample number was sufficient to permit the algorithm to converge on a global solution.

Maximum likelihood (ML) analyses were carried out using PAUP* v4.0b10 (Swofford, 2002). Model selection procedures implemented in Modeltest v3.06 (Posada and Crandall, 1998) identified the GTR+I+Γ model (general-time-reversible model (Yang, 1994a) with invariable sites (Hasegawa et al., 1985) and gamma-distributed rates (Yang, 1994b) for variable sites as the best fitting model from among 56 nested models. ML parameters were optimized using the BI consensus tree topology. With model

parameters estimated from the data, a heuristic search with three random taxon addition sequences, TBR branch swapping, and steepest descent option active was performed. Bootstrap analyses were conducted using 100 resampling replicates as implemented in PAUP*. For each bootstrap replicate, a heuristic search with three random taxon addition sequences, NNI branch swapping, and steepest descent option active was performed.

Estimating divergence times

Divergence times within the Charophyta were estimated using a Bayesian relaxed clock approach developed by Thorne and Kishino (2002) with the software DIVTIME 5b kindly provided by Dr. Jeffrey Thorne. This approach combines the advantages of relaxing the molecular clock with a continuous autocorrelation of substitution rates over evolutionary time and allows the simultaneous use of several calibration references. Calibration references were derived from the literature or with the generous assistance of Dr. Monique Feist (Table III-2). All absolute ages were taken from the 1999 Geological Time Scale of the Geological Society of America (www.geosociety.org/science/timescale/timescl.pdf).

The dating procedure involved two steps. First, the program ESTBRANCHES was used to estimate branch lengths and the variance-covariance matrix for each gene separately. The tree topology generated by the BI and ML analyses was used as well as an alternative topology that constrained *Chara* and Nitelleae to be monophyletic. This constrained topology, though not found by the BI or ML analyses, represents relationships based on morphology and used extensively in the Charales literature (Wood, 1965). The F84+Γ nucleotide substitution model is the most general model implemented in ESTBRANCHES, and this model was used for all dating analyses. Free parameters in

the F84+Γ model include base composition, transition-transversion rate parameter (Ti/Tv), and four values that correspond to each discrete gamma rate category (Γ_1-Γ_4). All parameters were estimated with PAUP* 4.0b10 and PAML 3.13 for individual gene data sets and can be found in Table III-3.

In the second step of dating, *Cyanophora paradoxa* (the outgroup) was pruned from the tree and the program DIVTIME was used to estimate the posterior and prior estimates of divergence ages, their standard deviations (SD), and 95% credibility intervals ($Cred_{95}$). For each analysis, the Markov chain was run for 2,200,000 generations. The first 200,000 generations were treated as burn-in and not saved. After the burn-in cycle, the chain was sampled every 100 generations for a total of 20,000 samples. The following prior distributions were set: 1 billion years (SD = 500 mya) for the expected time between tip and root if there had been no constraint on node times, 0.5 (SD = 0.25) substitutions per site for rate at root node, 1.5 (SD = 1.5) for the parameter 'nu' that controls the amount of rate autocorrelation, and 10 billion years for the highest number of time units between tip and root. To insure that the Markov chain reached stationarity, results from two independent runs with unique randomly selected starting points were compared for each analysis. Posterior and prior age estimates were compared to evaluate whether the final age estimates were predominately a result of the data or the priors.

Table III-2. List of constrained lineages with geologic age, upper and lower fossil calibration dates, and references used to estimate divergence times.

Constrained lineage	Geologic Age	MYA	Reference
Chara	Coniacian-Turonian	85-95	M. Feist pers. comm.
Lamprothamnium	Campanian-Coniacian	80-90	M. Feist pers. comm.
Nitellopsis obtusa	Calabrian	0.5-0.7	(Groves and Bullock-Webster, 1924)
Nitella	Albian	99-112	(Horn af Rantzien, 1957)
Tolypella sect. Tolypella	Maastrichtian	65-71.3	(Uliana and Musacchio, 1978)

TABLE III-3. Estimated-parameter values under the F84 model of sequence evolution as implemented in ESTBRANCHES. These include base composition (%), transition-transversion rate parameter (Ti/Tv), alpha (α), and four values that correspond to each discrete gamma rate category (Γ_1-Γ_4). Parameters were estimated with PAUP* 4.0b10 or PAML 3.13.

	% A	% C	% G	% T	Ti/Tv	alpha (α)	Γ_1	Γ_2	Γ_3	Γ_4
atpB	0.37588	0.39233	0.10557	0.27498	2.74744	0.28275	0.00400	0.09116	0.56566	3.33917
rbcL	0.29656	0.17722	0.11728	0.40894	1.55005	0.23518	0.00149	0.05621	0.46917	3.47313
nad5	0.25399	0.18803	0.14426	0.41372	1.36855	0.46225	0.02662	0.22626	0.79073	2.95608
SSU	0.21944	0.24616	0.26048	0.27392	1.65570	0.23096	0.00134	0.05333	0.45957	3.48576

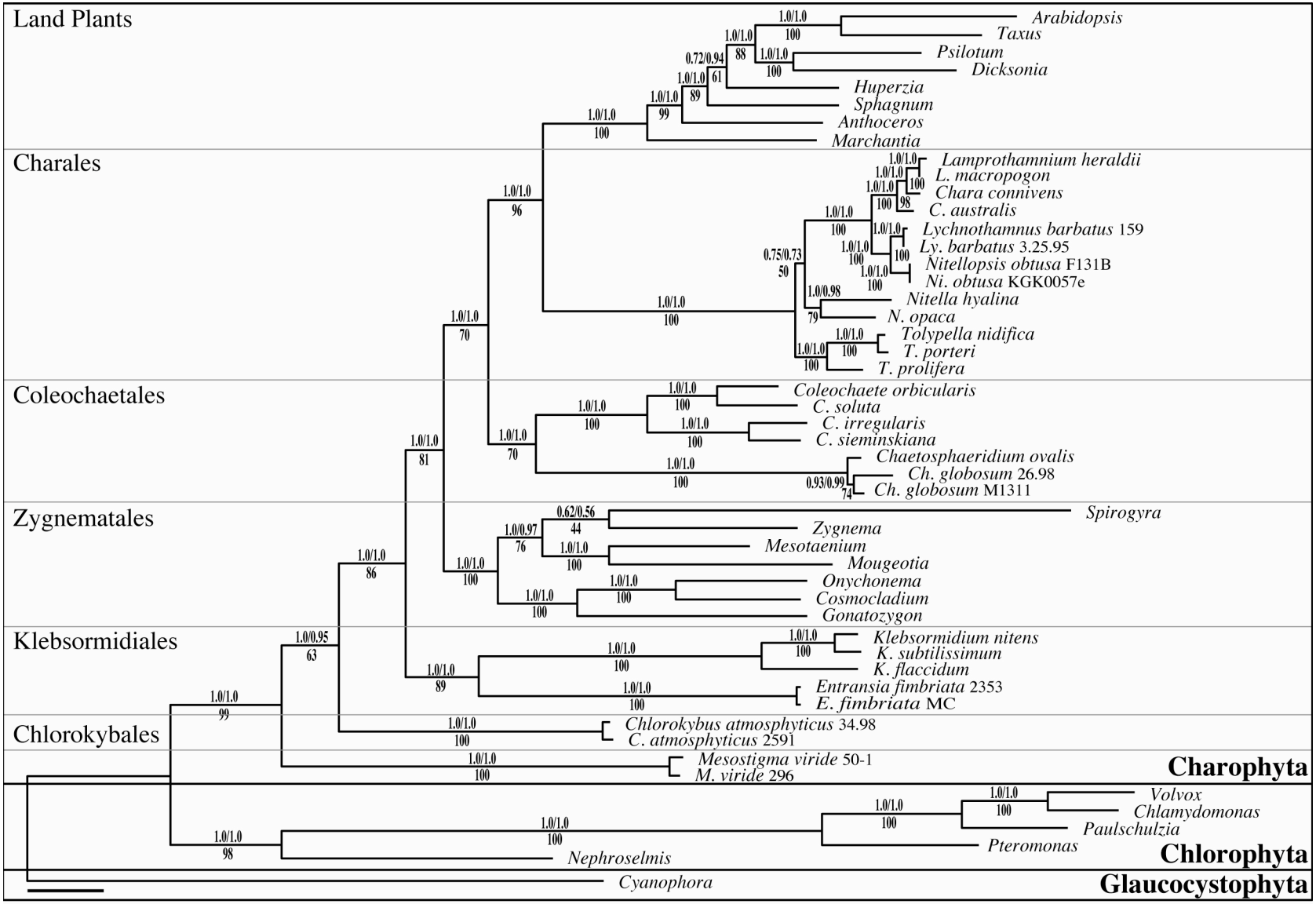
Results

Phylogenetic analyses

The data set used for phylogenetic analyses and estimating divergence times excluded introns and unalignable regions for 5,199 characters. A single highly variable region of *nad5* and three regions of the nuclear SSU gene, all of which corresponded to putative loop regions, were excluded. For the combined data set and for each gene separately, the best fitting model of sequence evolution as determined with Modeltest v3.06 was the general-time-reversible model with invariable sites and a gamma distributed correction for rate variation among variable sites (GTR+I+Γ). During iterative parameter estimation procedures (Swofford et al., 1996), parameters converged on identical values after two iterations and these values were fixed for all ML analyses.

Identical topologies were found by the BI and ML analyses of the combined four-gene data set. This topology, along with BI posterior probabilities using a single model (PP), BI posterior probabilities using four models (PP_4), and ML bootstrap values (BS), is presented in Figure III-1. Consistent with results presented in Chapter II, the Charales were monophyletic (BS=100, PP=1.0, PP_4=1.0) as were the land plants (BS=100, PP=1.0, PP_4=1.0). The Charales were found as sister to the land plants with strong support (BS=96, PP=1.0, PP_4=1.0). A monophyletic order Coleochaetales (BS=70, PP=1.0, PP_4=1.0) was found as sister to the Charales/land plant clade (BS=70, PP=1.0, PP_4=1.0). Other classically recognized orders were also found to be monophyletic: Zygnematales, BS=100, PP=1.0, PP_4=1.0; and Chlorokybales represented in this study by two strains, BS=100, PP=1.0, PP_4=1.0. The placement of a second strain of *Entransia fimbriata*

FIGURE III-1. Phylogenetic relationships for the Charophyta determined by Bayesian inference (BI) and maximum likelihood (ML) analyses using the combined four-gene data set rooted with *Cyanophora*. BI posterior probabilities using a single model (PP), and four models (PP_4) are presented above branches, respectively. ML bootstrap values for 100 replicates are presented below branches. All analyses resulted in an identical tree topology. For PP the likelihood of the best state drawn from the "cold" chain was -ln = 67847.95, for PP_4 -ln = 67163.76, and the maximum likelihood tree score was -ln = 67819.00. Branch lengths are Bayesian mean values from the PP_4 analysis and are proportional to the number of substitutions per site (scale bar, 0.05 substitutions/site). Taxonomy is modified from Mattox and Stewart (1984).


Land Plants
Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Charales
Lamprothamnium heraldii
L. macropogon
Chara connivens
C. australis
Lychnothamnus barbatus 159
Ly. barbatus 3.25.95
Nitellopsis obtusa F131B
Ni. obtusa KGK0057e
Nitella hyalina
N. opaca
Tolypella nidifica
T. porteri
T. prolifera
Coleochaetales
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum 26.98
Ch. globosum M1311
Zygnematales
Spirogyra
Zygnema
Mesotaenium
Mougeotia
Onychonema
Cosmocladium
Gonatozygon
Klebsormidiales
Klebsormidium nitens
K. subtilissimum
K. flaccidum
Entransia fimbriata 2353
E. fimbriata MC
Chlorokybales
Chlorokybus atmosphyticus 34.98
C. atmosphyticus 2591
Mesostigma viride 50-1
M. viride 296
Charophyta
Volvox
Chlamydomonas
Paulschulzia
Pteromonas
Nephroselmis
Chlorophyta
Cyanophora
Glaucocystophyta

sister to a UTEX strain of the same species (BS=100, PP=1.0, PP_4=1.0) confirmed the placement of this alga with the Klebsormidiales (BS=89, PP=1.0, PP_4=1.0). All analyses strongly support the inclusion of *Mesostigma* within the Charophyta (BS=99, PP=1.0, PP_4=1.0).

Within the Charales, the two sections of *Tolypella* formed a strongly supported monophyletic genus (BS=100, PP=1.0, PP_4=1.0) sister to the rest of the family. The tribe Nitelleae was not recovered, with a monophyletic *Nitella* (BS=79, PP=1.0, PP_4=0.98) found sister to the tribe Chareae (BS=100, PP=1.0, PP_4=1.0), rather than sister to *Tolypella* as suggested by classical treatments (Wood, 1965). The clade containing *Nitella* and Chareae is the only relationship within the Charales not strongly supported by any analytical method (BS=50, PP=0.75, PP_4=0.73). Within the Chareae two monophyletic pairs were recovered: *Nitellopsis*/*Lychnothamnus* (BS=100, PP=1.0, PP_4=1.0) and *Chara*/*Lamprothamnium* (BS=100, PP=1.0, PP_4=1.0). *Nitellopsis obtusa* (BS=100, PP=1.0, PP_4=1.0) and *Lychnothamnus barbatus* (BS=100, PP=1.0, PP_4=1.0) are each monophyletic, while *Chara* is paraphyletic (BS=98, PP=1.0, PP_4=1.0) with respect to a monophyletic *Lamprothamnium* (BS=100, PP=1.0, PP_4=1.0).

Comparisons of posterior probabilities between the Bayesian analyses using a single model of sequence evolution and four models revealed few differences (values above branches in Figure III-1). In general, nodes that received PP=1.0 in the single-model analysis received identical values for the four-model analysis (PP_4=1.0). Six comparisons did differ, although five of these differed only slightly ([Embryo...Chlorokybales], PP=1.0, PP_4=0.95; [Mesota...Zygnem], PP=1.0, PP_4=0.97; [Spirog...Zygnem], PP=0.62, PP_4=0.59; Chareae + *Nitella*, PP=0.75, PP_4=0.73; *Nitella*,

PP=1.0, PP_4=0.98). The sixth, and only substantial difference, was found for the vascular plant clade (i.e., tracheophytes, PP=0.72, PP_4=0.94).

Estimating divergence times

The Bayesian method of Thorne and Kishino (2002) was used to estimate divergence times within the Charophyta. Five calibration constraints were used simultaneously with two tree topologies, the best BI-ML tree topology and the constrained tree topology described above. For each topology, a relaxed molecular clock and a strict molecular clock were implemented. Comparisons of the posterior and prior divergence ages for each run showed that the 95% credibility intervals differ for most nodes, with narrower credibility intervals for posterior ages indicating that much of the information regarding divergence times can be attributed to the DNA sequence data rather than the priors (Table III-4a, Table III-4b). The constrained tree yielded generally older age estimates and slightly larger 95% credibility intervals than those generated using the BI-ML tree. Results generated under a strict molecular clock were markedly older than the relaxed clock analyses.

Table III-4. Estimated divergence times within the Charophyta. Five calibration points were used to estimate absolute divergence ages (Table III-2). Values correspond to the posterior and prior divergence age's ± one standard deviation. Values were calculated according to the Bayesian relaxed molecular clock approach and according to a strict molecular clock. The 95% credibility intervals ($Cred_{95}$) for posterior and prior divergence ages are given in parentheses. Two slightly different topologies were examined: **(a)** the BI-ML topology (Figure III-1), and **(b)** monophyletic *Chara* and monophyletic Nitelleae, respectively. Because valid names are not available for many of the deeper relationships (clades) within the Charophyta, names were created and are defined in Appendix I. Data are presented as million years ago (mya).

Table III-4a

Clade	Relaxed Clock		Strict Clock	
	Posterior	Prior	Posterior	Prior
Volvox...Chlam	213.02 ± 62.01 (114.30-354.92)	119.40 ± 119.56 (3.60-439.44)	439.19 ± 61.70 (327.00-569.32)	117.48 ± 116.94 (2.77-428.89)
Volvox...Paulsc	315.11 ± 79.93 (183.57-496.09)	240.11 ± 171.58 (32.55-675.07)	620.70 ± 77.74 (478.31-785.01)	235.30 ± 165.20 (30.53-659.85)
Volvox...Pterom	515.95 ± 105.47 (336.79-747.80)	361.44 ± 209.46 (85.08-887.77)	869.57 ± 101.82 (684.90-1087.18)	353.50 ± 203.57 (82.58-867.57)
Chlorophyta	928.56 ± 150.70 (668.57-1261.24)	479.71 ± 238.62 (157.72-1082.94)	1695.84 ± 186.33 (1359.06-2092.64)	471.71 ± 236.08 (155.62-1062.48)
Mesostigma	100.16 ± 58.64 (17.94-240.90)	271.42 ± 208.82 (12.70-787.48)	76.27 ± 16.17 (46.48-109.79)	269.77 ± 209.31 (11.94-799.79)
Chlorokybus	59.31 ± 36.70 (10.77-151.52)	243.90 ± 186.35 (11.15-708.27)	21.11 ± 9.70 (3.56-41.58)	241.63 ± 186.41 (10.48-709.19)
Entransia	4.33 ± 5.63 (0.12-19.98)	163.81 ± 139.16 (6.77-518.34)	6.08 ± 4.39 (0.28-16.55)	161.48 ± 136.22 (6.55-513.09)
Klenit...Klesub	60.73 ± 21.76 (29.35-112.77)	105.87 ± 102.55 (2.85-381.57)	178.20 ± 29.78 (123.42-240.52)	107.77 ± 103.59 (3.36-381.07)
Klenit...Klefla	211.01 ± 51.00 (128.46-324.89)	215.52 ± 142.36 (31.72-581.05)	551.43 ± 80.59 (407.53-720.64)	214.95 ± 143.13 (33.67-578.86)
Klebsormidiales	752.30 ± 119.74 (545.25-1014.28)	324.61 ± 172.50 (93.59-761.78)	1902.78 ± 205.64 (1535.25-2339.63)	322.28 ± 169.42 (92.14-749.34)
Mesota...Mougeo	426.80 ± 80.03 (289.10-600.73)	92.99 ± 88.11 (2.49-328.98)	1017.55 ± 122.78 (797.97-1281.83)	92.88 ± 89.13 (2.69-327.13)
Spirog...Zygnem	568.25 ± 94.70 (403.96-773.07)	94.09 ± 91.80 (2.47-342.96)	1143.32 ± 126.63 (915.68-1412.58)	93.23 ± 90.43 (2.59-332.03)
Mesota...Zygnem	625.86 ± 100.42 (451.79-844.19)	186.62 ± 124.95 (27.12-502.25)	1488.04 ± 152.65 (1219.09-1810.69)	186.54 ± 123.98 (27.98-500.38)
Cosmoc...Onycho	351.22 ± 71.51 (229.29-506.36)	93.31 ± 89.01 (2.71-326.15)	601.24 ± 77.45 (460.97-763.71)	93.11 ± 88.79 (2.53-325.12)
Cosmoc...Gonato	549.60 ± 93.33 (390.39-754.97)	188.54 ± 123.24 (28.33-498.16)	1171.18 ± 131.09 (937.95-1451.51)	187.56 ± 124.86 (26.75-503.43)
Zygnematales	706.32 ± 110.28 (515.32-946.80)	282.21 ± 149.95 (82.51-650.91)	1676.27 ± 165.70 (1383.90-2034.21)	280.60 ± 147.78 (78.53-653.28)
Chaetosphaeridium globosum	53.28 ± 23.06 (22.98-110.49)	80.85 ± 76.57 (2.63-280.50)	76.95 ± 16.76 (45.55-111.21)	79.62 ± 75.23 (2.35-272.88)
Chaetosphaeridium	64.32 ± 26.89 (28.71-131.09)	162.57 ± 105.70 (25.01-428.83)	112.33 ± 19.70 (75.93-153.28)	159.78 ± 103.95 (24.56-417.38)
Cirreg...Csiemi	220.35 ± 54.74 (130.39-342.61)	80.81 ± 76.22 (2.45-278.79)	348.3 ± 48.83 (260.23-451.36)	80.32 ± 76.59 (2.34-281.56)
Corbic...Csolut	303.43 ± 64.11 (195.90-444.89)	81.34 ± 77.63 (2.42-285.45)	515.67 ± 64.39 (399.63-649.00)	80.42 ± 75.94 (2.43-278.16)

Clade	Relaxed Clock		Strict Clock	
	Posterior	Prior	Posterior	Prior
Coleochaete	475.40 ± 81.09 (336.95-653.94)	161.74 ± 105.95 (25.70-433.89)	840.27 ± 93.78 (671.41-1040.54)	160.10 ± 103.69 (24.50-421.76)
Coleochaetales	668.50 ± 102.43 (491.83-891.22)	243.98 ± 126.57 (73.44-569.36)	1444.33 ± 151.59 (1173.86-1768.24)	239.94 ± 123.13 (73.47-553.28)
Tolypella sect. Tolypella	67.32 ± 1.70 (65.08-70.92)	68.13 ± 1.81 (65.16-71.14)	68.14 ± 1.81 (65.17-71.14)	68.13 ± 1.82 (65.16-71.15)
Tolypella	189.43 ± 24.01 (147.27-241.59)	140.08 ± 62.53 (70.80-307.03)	237.98 ± 37.06 (170.22-316.38)	139.57 ± 63.21 (70.73-303.53)
Nitella	109.62 ± 2.24 (103.59-111.94)	105.30 ± 3.72 (99.29-111.61)	107.5 ± 3.43 (99.95-111.85)	105.35 ± 3.76 (99.30-111.67)
Nitellopsis	0.60 ± 0.06 (0.51-0.70)	0.60 ± 0.06 (0.51-0.69)	0.60 ± 0.06 (0.51-0.69)	0.60 ± 0.06 (0.50-0.69)
Lychnothamnus	3.10 ± 2.46 (0.13-9.24)	31.06 ± 29.54 (0.57-103.97)	5.07 ± 3.78 (0.19-14.10)	30.90 ± 29.25 (0.54-103.24)
Lychno...Nitops	70.77 ± 14.06 (46.30-101.00)	61.90 ± 40.20 (3.75-148.92)	101.43 ± 20.54 (62.25-143.00)	61.75 ± 40.24 (3.63-148.20)
Lamprothamnium	81.37 ± 1.31 (80.04-84.87)	83.52 ± 2.53 (80.13-89.09)	81.27 ± 1.24 (80.03-84.57)	83.51 ± 2.54 (80.13-89.11)
Lampro...Cconni	88.24 ± 3.36 (81.66-93.86)	87.40 ± 3.16 (81.56-93.35)	87.05 ± 3.45 (81.10-93.52)	87.38 ± 3.15 (81.57-93.26)
Lampro...Caustr	92.40 ± 2.18 (86.97-94.92)	91.30 ± 2.59 (85.77-94.84)	91.92 ± 2.43 (86.22-94.90)	91.24 ± 2.60 (85.80-94.85)
Chareae	155.47 ± 16.32 (126.87-191.16)	122.72 ± 33.49 (91.09-214.21)	208.81 ± 29.74 (153.72-270.69)	122.80 ± 34.03 (90.93-212.69)
Chareae+*Nitella*	204.36 ± 18.14 (172.92-243.86)	163.50 ± 54.81 (106.68-309.05)	311.65 ± 37.30 (243.91-389.13)	163.50 ± 56.62 (106.68-316.76)
Charales	247.75 ± 25.98 (202.01-303.96)	212.23 ± 81.62 (118.09-424.13)	378.63 ± 47.83 (291.77-480.56)	211.64 ± 82.23 (118.27-430.33)
Moniliformopses	263.36 ± 48.63 (180.68-370.86)	37.99 ± 39.40 (0.96-142.32)	967.33 ± 113.16 (763.38-1207.52)	37.98 ± 38.84 (0.89-142.31)
Seed plants	230.88 ± 43.85 (156.78-328.10)	38.57 ± 39.68 (0.95-143.78)	969.42 ± 114.24 (766.12-1210.23)	37.97 ± 39.82 (1.02-141.57)
Euphyllophytes	341.44 ± 58.59 (242.05-469.67)	76.45 ± 56.18 (9.09-220.62)	1166.61 ± 113.50 (965.19-1407.69)	76.13 ± 56.39 (9.92-221.47)
Tracheophytes	389.92 ± 64.71 (279.19-528.94)	114.67 ± 69.60 (23.94-292.79)	1202.47 ± 114.44 (1000.77-1445.84)	114.25 ± 70.26 (25.54-293.61)
Trache...Sphagu	418.00 ± 68.07 (301.55-564.80)	152.95 ± 81.48 (45.29-361.21)	1234.31 ± 116.40 (1028.54-1480.77)	152.64 ± 81.98 (46.65-360.14)
Trache...Anthos	460.12 ± 72.56 (334.69-615.96)	191.15 ± 91.74 (70.44-423.51)	1288.33 ± 121.29 (1071.17-1546.51)	190.62 ± 93.18 (71.00-430.42)
(Land plants) Embryophytes	497.78 ± 75.66 (366.89-661.00)	229.45 ± 101.6 (99.71-485.26)	1359.89 ± 130.51 (1128.98-1638.14)	228.40 ± 102.86 (100.43-497.70)
Embryo...Charales	674.15 ± 99.69 (501.06-889.85)	267.91 ± 111.02 (131.57-551.65)	1747.99 ± 170.33 (1443.91-2112.97)	266.21 ± 112.27 (133.82-560.67)

	Relaxed Clock		Strict Clock	
Clade	Posterior	Prior	Posterior	Prior
Embryo...Coleochaetales	723.89 ± 109.16 (535.32-962.22)	324.43 ± 143.48 (147.83-692.54)	1858.65 ± 176.89 (1544.34-2239.97)	320.77 ± 139.65 (152.46-687.46)
Embryo...Zygnematales	775.87 ± 118.27 (571.03-1035.08)	378.10 ± 169.40 (166.40-802.05)	2013.19 ± 188.95 (1680.02-2420.64)	375.61 ± 166.28 (171.08-806.23)
Embryo...Klebsormidiales	826.92 ± 127.94 (605.99-1107.58)	433.15 ± 194.21 (185.83-921.75)	2107.04 ± 198.91 (1755.45-2532.86)	430.02 ± 192.05 (188.46-930.00)
Embryo...*Chlorokybus*	928.64 ± 143.71 (681.71-1247.18)	488.55 ± 218.32 (204.98-1043.64)	2142.42 ± 202.22 (1788.33-2572.98)	484.36 ± 217.11 (208.02-1045.55)
Charophyta	1003.9 ± 153.82 (740.52-1344.59)	543.07 ± 242.82 (226.42-1162.09)	2218.10 ± 211.34 (1844.61-2667.71)	538.18 ± 240.31 (226.84-1150.06)
Charophyta...Chlorophyta	1144.6 ± 173.72 (843.90-1526.79)	598.26 ± 266.44 (245.97-1273.82)	2526.63 ± 251.13 (2078.54-3059.72)	592.82 ± 264.89 (248.93-1262.16)

Table III-4b

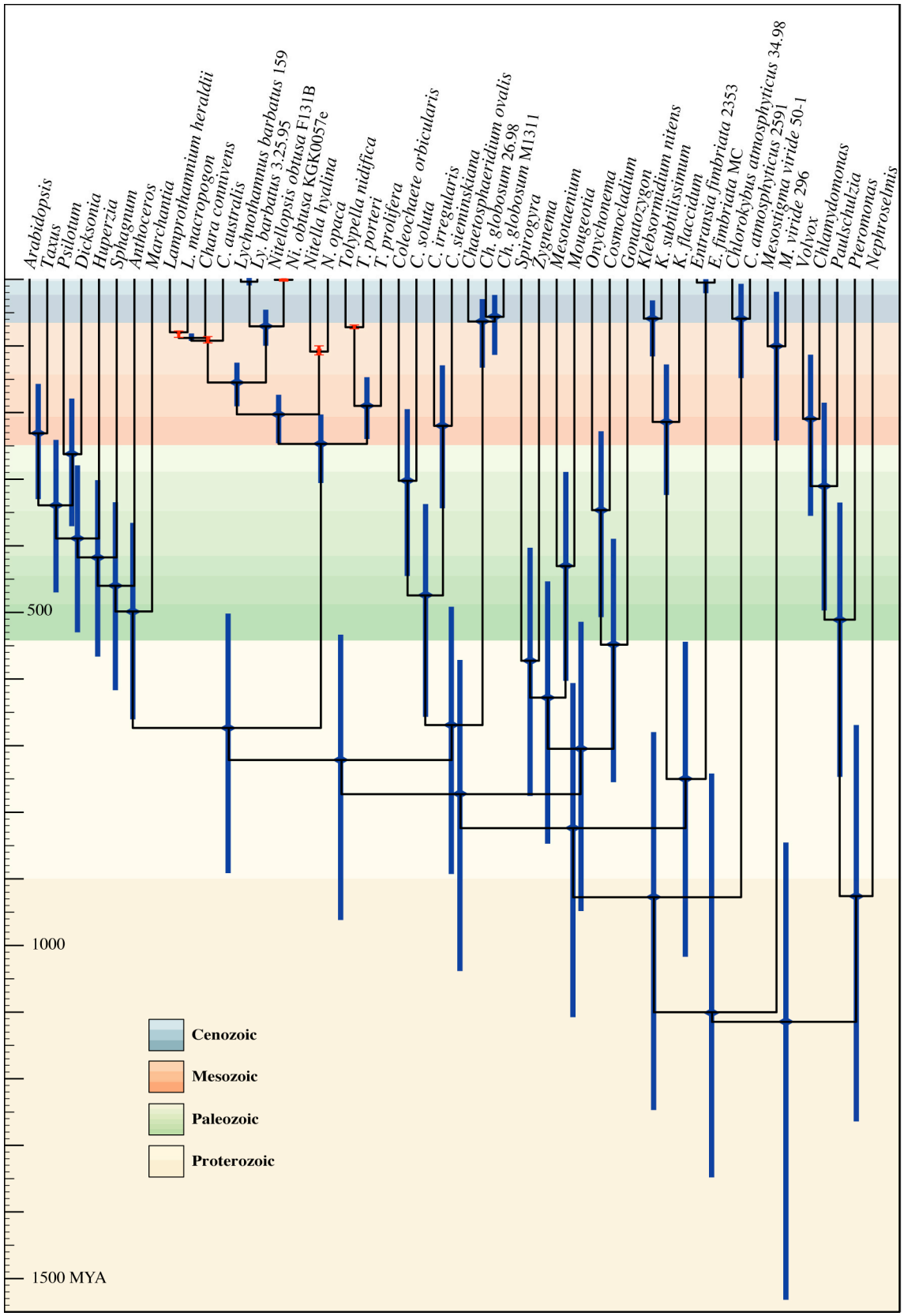
	Relaxed Clock		Strict Clock	
Clade	Posterior	Prior	Posterior	Prior
Volvox...Chlam	231.72 ± 65.44 (125.34-380.30)	146.79 ± 144.36 (3.99-535.55)	447.71 ± 62.84 (332.56-580.49)	150.93 ± 149.14 (4.10-553.14)
Volvox...Paulsc	344.23 ± 84.81 (203.51-533.71)	298.28 ± 203.09 (40.07-804.36)	636.16 ± 79.94 (492.04-805.34)	302.60 ± 212.48 (39.47-857.53)
Volvox...Pterom	550.65 ± 111.42 (363.24-797.22)	448.41 ± 246.84 (106.92-1043.84)	887.41 ± 103.94 (700.83-1106.44)	455.85 ± 264.13 (107.57-1115.50)
Chlorophyta	982.66 ± 160.16 (714.15-1344.78)	597.13 ± 278.60 (201.95-1279.52)	1733.91 ± 188.14 (1399.71-2133.98)	607.54 ± 305.87 (193.47-1369.31)
Mesostigma	113.62 ± 63.00 (23-267.40)	339.75 ± 254.32 (14.39-960.65)	75.97 ± 16.10 (45.98-109.69)	344.96 ± 268.73 (13.72-1027.25)
Chlorokybus	63.03 ± 39.43 (11.57-163.91)	311.48 ± 236.64 (14.39-891.86)	20.64 ± 9.62 (3.26-40.74)	316.60 ± 244.46 (13.02-929.33)
Entransia	4.65 ± 6.00 (0.12-21.30)	212.94 ± 176.54 (6.85-658.28)	5.88 ± 4.23 (0.25-15.77)	211.32 ± 180.48 (7.54-673.25)
Klenit...Klesub	64.81 ± 22.66 (32.04-118.92)	138.78 ± 130.80 (4.47-479.12)	180.65 ± 30.04 (125.47-243.50)	140.64 ± 136.22 (3.82-499.06)
Klenit...Klefla	225.38 ± 53.15 (139.19-345.47)	279.95 ± 179.76 (43.08-724.60)	562.91 ± 80.68 (417.09-731.59)	280.79 ± 189.64 (40.58-764.91)
Klebsormidiales	799.46 ± 127.12 (586.46-1084.36)	420.64 ± 210.51 (125.75-931.20)	1937.27 ± 206.96 (1564.10-2373.02)	423.01 ± 228.20 (114.14-997.00)
Mesota...Mougeo	452.87 ± 85.07 (308.12-639.92)	124.06 ± 115.92 (3.49-425.94)	1038.89 ± 124.74 (817.33-1300.68)	125.36 ± 123.01 (3.41-455.04)
Spirog...Zygnem	599.29 ± 100.23 (432.06-824.43)	121.93 ± 112.26 (3.35-413.06)	1147.74 ± 128.8 (919.01-1420.50)	126.70 ± 123.73 (3.62-453.69)
Mesota...Zygnem	664.14 ± 106.71 (487.07-903.63)	243.91 ± 153.82 (35.20-618.08)	1516.33 ± 154.07 (1246.19-1848.83)	251.09 ± 170.22 (36.54-685.78)
Cosmoc...Onycho	376.45 ± 75.94 (249.52-545.48)	123.91 ± 116.30 (3.32-435.61)	617.10 ± 79.73 (475.52-787.33)	123.77 ± 120.50 (3.51-441.32)
Cosmoc...Gonato	583.16 ± 98.17 (419.00-800.06)	246.15 ± 159.04 (37.09-642.01)	1200.71 ± 135.53 (961.45-1491.24)	248.41 ± 167.77 (36.24-676.11)
Zygnematales	748.81 ± 117.03 (554.07-1009.38)	366.91 ± 183.81 (104.42-815.48)	1709.50 ± 167.88 (1418.97-2071.39)	374.28 ± 203.40 (104.36-889.98)
Cheat. globosum	55.87 ± 24.44 (24.43-118.38)	108.23 ± 102.81 (3.30-375.61)	78.02 ± 16.64 (46.65-112.69)	107.86 ± 105.90 (3.08-386.06)
Chaetosphaeridium	67.16 ± 28.46 (29.95-139.01)	215.47 ± 139.13 (32.09-556.89)	113.36 ± 19.56 (77.78-154.08)	217.16 ± 147.37 (33.49-589.48)
Cirreg...Csiemi	234.90 ± 58.42 (139.72-366.51)	107.73 ± 103.13 (3.11-376.14)	352.88 ± 48.68 (264.46-453.91)	107.47 ± 104.68 (2.71-387.31)
Corbic...Csolut	323.31 ± 67.89 (210.23-475.11)	108.65 ± 103.15 (3.33-380.01)	522.09 ± 65.07 (403.80-660.61)	108.71 ± 106.22 (3.08-391.65)

Clade	Relaxed Clock		Strict Clock	
	Posterior	Prior	Posterior	Prior
Coleochaete	505.21 ± 85.89 (360.24-696.68)	216.06 ± 140.90 (33.76-562.37)	858.06 ± 95.01 (687.08-1061.63)	216.18 ± 145.33 (32.66-586.04)
Coleochaetales	710.49 ± 108.64 (531.17-954.08)	322.23 ± 166.12 (93.89-730.32)	1485.50 ± 154.64 (1211.38-1817.19)	325.08 ± 177.16 (95.03-774.41)
Tolypella sect. Tolypella	67.17 ± 1.66 (65.07-70.85)	68.13 ± 1.83 (65.15-71.14)	68.08 ± 1.81 (65.15-71.12)	68.11 ± 1.82 (65.15-71.13)
Tolypella	166.01 ± 20.55 (129.92-210.29)	130.36 ± 57.37 (70.45-284.25)	210.95 ± 34.20 (147.37-280.66)	130.75 ± 60.87 (70.43-293.50)
Nitella	109.72 ± 2.16 (103.73-111.94)	105.39 ± 3.71 (99.35-111.64)	107.46 ± 3.43 (99.88-111.85)	105.42 ± 3.75 (99.33-111.67)
Nitelleae	215.37 ± 20.15 (180.85-259.74)	192.22 ± 78.35 (108.65-402.69)	312.57 ± 38.99 (240.98-394.14)	192.62 ± 83.24 (108.11-418.44)
Nitellopsis	0.60 ± 0.06 (0.51-0.69)	0.60 ± 0.06 (0.51-0.69)	0.60 ± 0.06 (0.50-0.70)	0.60 ± 0.06 (0.51-0.70)
Lychnothamnus	3.84 ± 3.12 (0.15-11.71)	46.90 ± 47.93 (0.81-170.58)	5.15 ± 3.78 (0.21-14.16)	47.06 ± 48.83 (0.77-173.50)
Lychno...Nitops	91.53 ± 17.90 (60.02-130.26)	95.04 ± 69.46 (4.98-265.79)	113.21 ± 20.97 (74.11-156.44)	94.38 ± 70.68 (4.53-266.63)
Lamprothamnium	81.99 ± 1.87 (80.05-87.13)	84.96 ± 2.88 (80.26-89.72)	81.64 ± 1.61 (80.04-86.08)	84.97 ± 2.89 (80.25-89.75)
Chara	90.97 ± 2.76 (85.48-94.85)	89.82 ± 2.85 (85.22-94.72)	90.80 ± 2.82 (85.39-94.85)	89.82 ± 2.89 (85.24-94.75)
Chara...Lampro	122.51 ± 9.76 (104.71-142.92)	131.65 ± 46.78 (89.89-259.85)	140.14 ± 16.47 (109.26-174.23)	131.71 ± 46.63 (89.79-257.96)
Chareae	187.75 ± 20.43 (151.6-231.06)	188.44 ± 77.56 (101.10-395.86)	250.00 ± 32.42 (190.13-317.86)	189.06 ± 81.46 (101.67-399.79)
Charales	277.98 ± 28.21 (229.23-340.26)	263.44 ± 106.86 (132.96-543.74)	404.85 ± 49.97 (312.85-509.24)	266.69 ± 117.10 (131.02-573.71)
Moniliformopses	282.14 ± 51.17 (197.51-394.44)	51.01 ± 51.50 (1.40-186.19)	986.33 ± 115.27 (780.00-1230.55)	52.16 ± 55.56 (1.33-201.19)
Seed plants	245.80 ± 46.06 (168.77-348.20)	51.73 ± 53.82 (1.26-193.23)	984.75 ± 114.92 (777.78-1227.84)	52.27 ± 55.34 (1.25-198.98)
Euphyllophytes	362.74 ± 61.63 (259.73-501.37)	102.54 ± 75.80 (12.59-295.54)	1183.88 ± 115.14 (979.65-1431.00)	104.40 ± 81.08 (12.32-318.93)
Tracheophytes	414.89 ± 68.11 (301.19-566.08)	154.11 ± 94.06 (31.80-385.71)	1219.48 ± 115.95 (1013.14-1469.56)	157.21 ± 103.56 (31.92-429.30)
Trache...Sphagu	445.48 ± 71.84 (325.24-606.03)	206.32 ± 111.06 (58.87-476.91)	1251.97 ± 118.23 (1042.48-1504.38)	210.12 ± 122.34 (58.52-526.73)
Trache...Anthos	490.37 ± 76.43 (361.57-660.84)	258.34 ± 125.38 (89.88-572.5)	1306.52 ± 122.75 (1089.14-1569.73)	262.79 ± 140.10 (89.83-624.69)
(Land plants) Embryophytes	526.31 ± 79.34 (391.98-703.50)	310.19 ± 139.23 (126.08-654.81)	1380.23 ± 132.66 (1146.58-1670.90)	314.59 ± 156.00 (124.53-715.48)
Embryo...Charales	716.74 ± 105.53 (539.02-952.27)	361.91 ± 152.65 (164.37-741.66)	1800.18 ± 174.54 (1491.50-2176.28)	366.93 ± 171.77 (160.05-809.29)

	Relaxed Clock		Strict Clock	
Clade	Posterior	Prior	Posterior	Prior
Embryo...Coleochaetales	771.24 ± 116.54 (576.67-1032.21)	427.20 ± 182.00 (188.92-879.80)	1910.86 ± 181.05 (1594.83-2300.75)	432.84 ± 201.49 (184.59-935.62)
Embryo...Zygnematales	825.70 ± 126.28 (614.87-1109.49)	491.09 ± 206.54 (214.43-1010.56)	2056.55 ± 191.40 (1719.54-2472.41)	498.73 ± 230.88 (206.74-1083.35)
Embryo...Klebsormidiales	878.10 ± 136.24 (649.92-1182.80)	555.07 ± 232.47 (240.19-1140.08)	2149.41 ± 200.82 (1794.36-2582.95)	563.86 ± 258.11 (231.94-1203.94)
Embryo...*Chlorokybus*	987.17 ± 153.09 (730.23-1328.51)	619.15 ± 257.48 (265.41-1260.62)	2185.15 ± 204.48 (1823.74-2631.15)	628.84 ± 286.71 (254.93-1350.64)
Charophyta	1063.34 ± 164.54 (787.83-1433.56)	682.75 ± 280.49 (291.77-1372.54)	2260.30 ± 213.64 (1883.78-2719.74)	693.89 ± 314.13 (280.96-1471.36)
Charophyta...Chlorophyta	1206.35 ± 184.95 (896.82-1621.47)	746.91 ± 305.62 (317.72-1494.33)	2582.46 ± 254.38 (2133.17-3128.13)	758.97 ± 341.06 (306.80-1607.64)

The individual *a priori* calibration constraints ranged in age from the Upper Early Cretaceous for *Nitella*, the Middle Late Cretaceous for *Chara* and *Lamprothamnium*, the uppermost Late Cretaceous for *Tolypella* section Tolypella, to the Early Quaternary (Pleistocene) for *Nitellopsis obtusa* (Table III-2). The Bayesian relaxed clock approach estimated that the Charophyta and Chlorophyta split occurred 1144.6 ± 173.72 MYA during the Middle Proterozoic, the lower bound of the prior 95% credibility interval. The lineage that gave rise to Mesostigmatales split shortly thereafter (1003.9 ± 153.82 MYA) followed by the Chlorokybales lineage (928.64 ± 143.71 MYA) from the remaining Charophyta. The Late Proterozoic represents a period of relatively rapid cladogenesis for the Charophyta resulting in the Klebsormidiales (752.3 ± 119.74 MYA), Zygnematales (706.32 ± 110.28 MYA), Coleochaetales (668.50 ± 102.43 MYA), and the divergence of the Charales/land plant common ancestor (674.10 ± 99.96 MYA). Modern land plants arose in the Cambrian (497.78 ± 75.66 MYA) and modern Characeae diverged at the Paleozoic/Mesozoic boundary (247.75 ± 25.98 MYA). These results are presented in tree form in Figure III-2.

Figure-III-2. BI-ML tree topology drawn with branch lengths proportional to absolute divergence times inferred from the Bayesian relaxed molecular clock approach. Red vertical bars represent upper and lower limits of fossil dates on constrained nodes, blue vertical bars show posterior 95% credibility intervals ($Cred_{95}$) for non-constrained nodes. The geologic time scale is shown as follows: Cenozoic, blue; Mesozoic, red; and Paleozoic, green. Shades of each color represent the Periods within those Eras. The Late and Middle Proterozoic are shown in shades of yellow.


Arabidopsis
Taxus
Psilotum
Dicksonia
Huperzia
Sphagnum
Anthoceros
Marchantia
Lamprothamnium heraldii
L. macropogon
Chara connivens
C. australis
Lychnothamnus barbatus 159
Ly. barbatus 3.25.95
Nitellopsis obtusa F131B
Ni. obtusa KGK0057e
Nitella hyalina
N. opaca
Tolypella nidifica
T. porteri
T. prolifera
Coleochaete orbicularis
C. soluta
C. irregularis
C. sieminskiana
Chaetosphaeridium ovalis
Ch. globosum 26.98
Ch. globosum M1311
Spirogyra
Zygnema
Mesotaenium
Mougeotia
Onychonema
Cosmocladium
Gonatozygon
Klebsormidium nitens
K. subtilissimum
K. flaccidum
Entransia fimbriata 2353
E. fimbriata MC
Chlorokybus atmosphyticus 34.98
C. atmosphyticus 2591
Mesostigma viride 50-1
M. viride 296
Volvox
Chlamydomonas
Paulschulzia
Pteromonas
Nephroselmis
500
1000
1500 MYA
Cenozoic
Mesozoic
Paleozoic
Proterozoic

The BI-ML tree topology was used to explore the relative contribution of each calibration constraint to the result using all calibration constraints simultaneously. These comparisons are presented in Figure III-3. Taken together, the 95% credibility intervals using a single calibration constraint overlap to form a continuous distribution that covers a relatively large time interval. These intervals were substantially reduced in the combined analysis. The 95% credibility intervals were generally wider for the single-calibration analyses with the exception of *Nitella,* which were generally narrower than other single-calibration analyses and the combined analysis. The 95% credibility intervals for *Lamprothamnium* were especially wide and were consistently found outside of the combined 95% credibility intervals.

Figure III-3. Comparison of the posterior 95% credible intervals. Using the BI-ML topology separate analyses were performed for the five calibration constraints. The 95% credibility intervals for these analyses (black bars) are shown along with results using all constraints simultaneously (grey bars). Individual results for each constraint are presented as follows: *Lamprothamnium*, *Tolypella*, [Lampro...Caustr], *Nitellopsis*, and *Nitella*. An asterisk (*) denoted constrained clades. Clades along the X-axis are in identical order as Table III-4a and the Y-axis is the time scale in billion years (BYA).

Volvox...Chlam
Volvox...Pauls
Volvox...Pleiom
Volvox...Chlorophyta
Mesostigma
Chlorokybus
Entransia
Klenit...Klesub
Klenit...Klella
Klebsormidiales
Mesota...Mousgeo
Spirog...Zygnem
Mesota...Zygnem
Cosmoc...Onycho
Cosmoc...Gonato
Zygnematales
Chaetosphaeridium globosum
Chaetosphaeridium
Cirteg...Csieni
Corbic...Csolut
Coleochaete
Coleochaetales
Tolypella sect. Tolypella
Tolypella
Nitella
Nitellopsis
Lychnothamnus
Lycchno...Nitops
Lamprothamnium
Lampro...Cconni
Lampro...Caustr
Charae+Nitella
Chareae
Charales
Moniliformopses
Seed plants
Euphyllophytes
Tracheophytes
Trache...Sphagn
Trache...Anthos
(Land plants)
Embryophytes
Embryo...Charales
Embryo...Coleochaetales
Embryo...Zygnematales
Embryo...Klebsormidiales
Embryo...Chlorokybus
Charophyta
Embryo...Chlorophyta
Charophyta...Chlorophyta
BYA
3.0
2.5
2.0
1.5
1.0
0.5
0.0

Discussion

Phylogenetic analyses

As in the analyses presented here, Chapter II included a range of charophycean algal diversity, but used data from fewer taxa particularly in the Charales. The higher-lever relationships shown in Chapter II (Figure II-2) were similar to those reported here (Figure III-1), with Charales sister to land plants and Coleochaetales sister to the Charales/land plant clade. The clades [Embryo...Zygnematales], Klebsormidiales, and [Mesota...Zygnem] showed increased bootstrap support which is unsurprising as new data from an additional strain of *Chlorokybus atmosphyticus* and *Entransia fimbriata* and *nad5* data for *Spirogyra maxima* were added to this data set. Support within the Chlorophyta also seemed to be improved by sampling sequences from genes in the same species (Chapter III) rather than sequences sampled from different species of the same genus (Chapter II). This result is, on the one hand, reassuring in that support values increased, but on the other hand, stresses the advantage of using sequences determined from the same species (if not the same individual) over concatenated genes from putative close relatives.

The additional representatives of the Charales had little impact on support for the Charales/land plant clade (the analyses in Chapter II and here show strong support), although, relationships within the Charales were interesting. The multi-gene analyses produced relationships differing in certain features from that proposed by Wood (1965), yet there were some shared features. Monophyly of the Charales was firmly established by these data with further support to be found in vegetative and reproductive morphology

(Wood, 1965) as well as the fossil record (Grambast, 1974). Monophyly of the tribe Chareae, united by five coronal cells, was also strongly supported, but monophyly of Nitelleae (*Tolypella+Nitella*) was not. A similar result was found using *rbcL* sequence data (McCourt et al., 1999; McCourt et al., 1996a). All members of the Nitelleae possess ten coronal cells and this character has been interpreted as a synapomorphy for the tribe. Although not strongly supported, the possibility exists that ten coronal cells represent an ancient ancestral character state rather than a character state derived in Nitelleae. The fossil record offers little resolution to this question because coronal cells are rarely preserved (Feist and Grambast-Fessard, 1991; Grambast, 1974; Tappan, 1980).

Within the Chareae, three of the four genera were monophyletic, *Nitellopsis*, *Lychnothamnus*, and *Lamprothamnium*. Both *Nitellopsis* and *Lychnothamnus* contain one species and are relatively rare (Wood, 1965). *Lamprothamnium* contains several species, and is cosmopolitan, but species in this genus generally are restricted to brackish environments (Corillion, 1957; Wood, 1965). *Chara,* represented by two species in this study, was paraphyletic with respect to *Lamprothamnium*, a result also found using nuclear SSU gene sequence data alone (Meiers et al., 1999). Characters distinguishing *Lamprothamnium* from *Chara* include elongate stipulodes found opposite branchlets, stipulodes sometimes above branchlets, incurved branchlets, upper whorls contracted into 'fox tails' resulting from reduced internode cells, and oogonia generally positioned below antheridia. In *Chara,* stipulodes are alternate and never found above branchlets, and oogonia are always found above antheridia (when conjoined). *Lamprothamnium* is entirely ecorticate as are members of *Chara* section Charopsis. Both male and female gametangia originate from the same peripheral cell in *Lamprothamnium* and *Chara* while

they initiate from different peripheral cells in *Lychnothamnus*. The dioecious state of *Nitellopsis* prevents examination of gametangia orientation and ontogeny in this genus. The taxonomic status of *Lamprothamnium* is unclear, and as suggested by Wood (1965), *Lamprothamnium* could well represent a third subgenus of *Chara*. Nuclear SSU gene sequence data placed *Lamprothamnium* sister to a clade including Charopsis (ecorticate) and Agardhia (axial cortication), though this result was sensitive to analytical method (Meiers et al., 1999). Phylogenetic analyses using *rbcL* (McCourt et al., 1999) or *rbcL* and *matK* (Sanders et al., 2003) placed *Lamprothamnium* sister to *Chara*; however, representatives of sections Charopsis or Agardhia were not included in either study. Results presented here are limited to only two *Chara* species, *Chara connivens* (section Grovesia) and *Chara australis* (section Charopsis). *Lamprothamnium* was found sister to *Chara connivens*, not sister to *Chara australis*, presenting yet another hypothesis. It is clear that further morphological and molecular data will be required to resolve the phylogenetic placement of *Lamprothamnium*.

'Simple' and 'complex' models

In Chapter II, a Bayesian analysis using a general model of DNA substitution (GTR+I+Γ) was applied to the entire four-gene data set. A similar analysis was performed here in addition to an analysis that partitioned the data by gene, in which GTR+I+Γ parameters were estimated separately for each partition. Comparisons of posterior probabilities between these analyses (Figure III-1) revealed several differences, all of which were minor with the exception of one. Several possibilities exist that could explain this result. On the one hand, the differences may represent a more realistic description of these data, i.e., that the topology is not entirely stable. Many millions of

years have pasted since the ancestor of the Charophyta diverged, and it is unlikely that any single model of DNA evolution can accurately describe these data without error. Likelihood methods, however, are generally robust to model violation (Felsenstein, 1981) and the limited number of differences between these analyses may be an example of this. The relatively large difference detected for the vascular plant clade (tracheophytes) is an especially noteworthy result (PP = 0.72, PP_4 = 0.94; Figure III-1). It is possible that some feature of a particular gene in this clade is evolving differently from the same gene in other lineages (e.g., long-branch-attraction, base compositional bias). If this is the case, parameters estimated across the entire dataset may not adequately describe the vascular plant clade data, leading to violation of the model. On the other hand, random error incurred by adding parameters must also be considered. Complex models are more sensitive to random error than simple models because more parameters are estimated from the same amount of data. The nodes that exhibit relatively small amounts of variation could be an example of this phenomenon. The vascular plant clade offers an interesting region of this tree to further study the underlying cause of this difference. The small number of differences between posterior probabilities across the entire tree suggests that both models might be performing similarly, and additional parameters may be useful in detecting regions of the tree requiring further study.

The fossil record of Characeae genera

Minimum fossil dates for five extant lineages of the Characeae were determined from the literature or with the assistance of Dr. Monique Feist (Montpellier) (Table III-X). Available field or culture collections of extant forms allowed fossil record comparisons of *Chara*, *Lamprothamnium*, and *Nitellopsis obtusa* for Chareae, and *Nitella*

and *Tolypella* section Tolypella for Nitelleae. Fossil calibration dates for *Chara* and *Lamprothamnium* (both Late Cretaceous) were provided by Dr. Monique Feist. The dioecious *Nitellopsis obtusa* is the only extant species of this genus. It is relatively rare and typically reproduces by specialized starch-rich bulbils. Consequently little was known of *Nitellopsis* oospores until Krassavina (1971) found oospores in the gut contents of ducks and from this was able to establish a connection of *Nitellopsis obtusa* with the fossil species *Tectochara diluviana*. The genus *Tectochara* (now considered a synonym of *Nitellopsis*) is known from the Paleocene to Quaternary, although *Nitellopsis obtusa* (*Tectochara diluviana*) has a more restricted fossil history (lowermost Quaternary from the Cromer Forest bed) (Groves, 1933; Groves and Bullock-Webster, 1924).

Nitella is one of the oldest and most species rich extant genera in the Characeae; however, *Nitella* fossils are relatively rare. Unlike other extant genera, *Nitella* has small oospores, which are relatively small, laterally compressed, and have spiral jacket-cells which do not calcify, leading to their paucity in the fossil record. Nevertheless, there are several reports of Quaternary and Pre-Quaternary gyrogonites assigned to *Nitella* [see Horn af Rantzien (1957; 1959) and references therein]. The gyrogonites' sizes, shapes, and lack of calcification were the main characters used to ally the Quaternary specimens with *Nitella*. The Pre-Quaternary gyrogonites have been judged as likely not belonging to *Nitella* since they show no characters unique to *Nitella*, and most notably are not laterally compressed (Groves, 1933; Horn af Rantzien, 1951; Horn af Rantzien, 1959). A remarkable exception to this trend was first reported by Vishnu-Mittre (1952) from the Rajmahal Basin, Bihar, India and formally described by Horn af Rantzien (1957). The Rajmahal gyrogonites are relatively small and laterally compressed but show well-

preserved oospore cell wall ornamentation, strikingly similar to many extant members of *Nitella*. In the absence of coronal cells and vegetative thalli that would unequivocally identify these fossils as *Nitella*, Horn af Rantzien (1957) was conservative and placed these fossils in a new organ genus *Nitellites*. The geologic age for the Rajmahal series has been difficult to determine with confidence and this is reflected in the scope of ages proposed for this region, from Triassic to early Cretaceous (summarized in Horn af Rantzien, 1957). This period of India's geologic history is complicated by the onset of volcanism that produced the Deccan traps (large flood basalts) and covered or wiped out much of the sedimentary record. Detailed stratigraphic mapping (Sen Gupta, 1988) combined with radiometric dating (Tiwari and Tripathi, 1993) and megaflora evidence (Banerji, 2000) now firmly establishes an Early Cretaceous (Albian) age for the *Nitellites* fossils.

The basal plate subtending the oospore is rarely found in the fossil record, but distinct basal plate impressions can clearly be identified on the oospore wall of fossil and extant forms. Grambast (1956) recognized the phylogenetic utility of this character to distinguish genera in the Characeae. Before that time it was thought that all members of Chareae possessed a simple basal plate (1-celled) and Nitelleae a multipartite (2 or 3-celled) basal plate. Subsequently, Daily (1969) showed that the basal plate could be either simple (as in Chareae) or multipartite (as in Nitelleae) in the living species of *Tolypella* and the distribution of this character was consistent with the sectional classification of the genus, *Tolypella* section Tolypella (= Obtusifolia) with a multipartite basal plate and section Rothia (= Acutifolia) with a simple basal plate. Sawa and Frame (1974) presented a survey of several *Tolypella* species confirming Daily's work and

proposed a hypothesis of basal plate development for the Characeae. With a clear understanding of extant *Tolypella* oospore morphology, it became possible to unite fossil and extant forms whereby the fossil genus *Sphaerochara*, with a simple basal plate, was placed in synonymy with *Tolypella* sect Rothia (Feist and Grambast-Fessard, 1982). Both sections of *Tolypella* are found in the fossil record, section Rothia (as *Sphaerochara*) from the Early Cretaceous (Hauterivian) (Wang, 1965) and section Tolypella from the Late Cretaceous (Maastrichtian) (Uliana and Musacchio, 1978).

Single versus multiple calibration points

The *a priori* calibration constraints discussed above ranged in age from the Upper Early Cretaceous for *Nitella* to the Early Quaternary (Pleistocene) for *Nitellopsis obtusa* (Table III-2). The BI-ML tree topology was used to explore the relative contribution of each calibration constraint to the result using all calibration constraints simultaneously (Figure III-X). The individual 95% credibility intervals for each clade overlapped to form a continuous distribution covering a relatively large time interval. These time intervals were substantially reduced for the combined analysis, an expected result given that the addition of more information should act to increase overall precision and accuracy. The analysis using the *Lamprothamnium* constraint alone produced extreme results, possibly due to an inaccurate fossil date, taxon sampling, and/or tree topology. An active debate exists in the literature regarding the delimitation of the genus *Lamprothamnium* in the fossil record. Both extant *Lamprothamnium* and fossil *Aclistochara* possess a deep apical peripheral grove, are cylindrical, and are unornamented. These characters lead Soulié-Märche (1979) to unite these genera, effectively extending the age of *Lamprothamnium* to the Jurassic. On the other hand,

Feist and Grambast-Fessard (1982; 1991) argued that the overall size and narrower apical zone of *Aclistochara* better resembles members of extinct Porocharaceae, and they maintained two distinct genera. If fossils of *Lamprothamnium* are not easily distinguishable, the calibration date used in this study may belong to an older distantly related lineage. *Lamprothamnium* has been recognized as a genus separate yet closely related to *Chara* (Wood, 1965); however, the molecular data place *Lamprothamnium* within *Chara* (see above). If *Lamprothamnium* is truly sister to *Chara* and not imbedded within it, then branch length reconstruction becomes an important consideration, as inaccurate topology and branch lengths would lead to inaccurate divergence time estimates. Both the BI-ML and constrained tree topologies force the absolute age of *Lamprothamnium* to the upper bound of the *a priori* distribution (Tables III-4a, III-4b), suggesting potential conflict between the fossil and molecular data. The individual analyses using *Nitellopsis*, *Tolypella*, and *Nitella* consistently estimated *Lamprothamnium* diverging more recently than interpreted in the fossil record (4.7 ± 2.5, 12.6 ± 6.0, 4.1 ± 1.8 MYA, respectively). It is also possible that the molecular data do not adequately represent extant *Lamprothamnium* diversity, effectively underestimating the age of the genus. Additional molecular data are needed to resolve the phylogenetic placement of *Lamprothamnium* with respect to *Chara* and a critical reassessment of the *Chara*/*Lamprothamnium* fossil record is necessary.

Proterozoic origin and diversification of the Charophyta

The Bayesian relaxed clock approach estimated the Charophyta/Chlorophyta split to occur 1,144.6 ± 173.72 MYA in the Middle Proterozoic. The lineage that gave rise to Mesostigmatales diverged shortly thereafter (1,003.9 ± 153.82 MYA) followed by

Chlorokybales (928.64 ± 143.71). The remaining major extant lineages of the Charophyta diverged in the Late Proterozoic including the split of the Charales/land plant common ancestor (674.15 ± 99.69) (Table III-4a, Figure III-2).

The Late Proterozoic represents a remarkable interval of evolutionary change with taxonomic diversity that far exceeds any earlier era (Knoll, 1996). Well-preserved fossils attributed to the red-algal bangiophyte lineage have been identified from the Hunting Formation, Somerset Island, Canada (1,250-750 MYA) (Butterfield et al., 1990). Other multicellular algae of uncertain taxonomic affinity are known from the mudstones of the Lakhanda Group, eastern Siberia (1,000-900 MYA) (Knoll, 1992; Knoll, 1996). The earliest putative unicellular and filamentous green algal fossils come from the Bitter Spring Formation, central Australia (~900 MYA) (Schopf, 1968; Schopf and Blacic, 1971). Somewhat younger shales from the Svanbergfjellet Formation, Spitsbergen, (~750 MYA) preserve algae with significant similarity to chlorophyte green algae such as *Cladophora* and *Coelastrum* (Butterfield et al., 1988). The oldest Charales fossils bear striking similarity to extant forms, which indicates that major features of the Charales were already established in the Silurian. Overall, it is not surprising that early cladogenesis in the Charophyta (Figure III-2) is found in the Late Proterozoic.

The rise of atmospheric oxygen in the Late Proterozoic has been attributed to increased cyanobacterial production and correlated with increased diversity of eukaryotic phototrophs, possibly leading to the Cambrian explosion of animals (Knoll, 1992). Results presented here make it possible to add another piece to this already complex puzzle and suggest that the charophyte algae also may have played a key ecological role during the Late Proterozoic.

Diversification of land plants

Consistent with the fossil record (Graham, 1993; Grey, 1993; Kenrick and Crane, 1997a) and other land plant molecular divergence times studies (Goremykin et al., 1997; Martin et al., 1993; Sanderson, 2003; Sanderson and Doyle, 2001), the results presented here suggest that extant land plants diverged 497.78 ± 75.66 MYA. Using protein sequences from fungi, animals and green plants, Heckman *et al*. (2001) presented a divergence time estimate of 703 ± 45 MYA for mosses and vascular plants. This result was particularly surprising not only because it is nearly 200 million years older than the oldest land plant fossil but also because, if hornworts and liverworts diverged before the moss/vascular plant clade (as suggested in Figure III-1), then the divergence time for the earliest land plants would be even older. Taxon sampling and choice of fossil constraints might explain this disparity (Sanderson, 2003). Heckman *et al*. (2001) included only one chlorophyte (*Chlamydomonas*) and no charophyte green algae and age constraints were derived from either the fungal fossil record or the crown group node of animals, plants, and fungi [which was in turn derived from a divergence time study of vertebrates (Wang et al., 1999)]. Though Sanderson (2003) found few differences between strict and relaxed clock analyses for 27 plastid proteins from land plants, reanalyzing the nuclear protein data used by Heckman *et al*. (2001) under a relaxed clock could result in younger age estimates. For example, strict clock estimates presented here were consistently older (Table III-4a, III-4b), and estimated the land plant divergence time at 526.31 ± 79.34 MYA, nearly 30 million years older than under the relaxed clock.

The Bayesian relaxed clock estimates presented here are generally congruent with other fossil information not used as calibration constraints. As mentioned above, the

oldest land plant microfossils are from the Ordovician and fall within the divergence time estimate (497.78 ± 75.66 MYA). The hornworts diverged 490.37 ± 76.43 MYA and the mosses diverged 418.00 ± 68.07 MYA, consistent with Early Silurian (latest Llandovery, ~432 MYA) to Early Devonian (mid Lochkovian, ~ 402 MYA) fossils of individually dispersed, simple spores, which are found in modern-day hornworts and some mosses (Grey, 1993). The Late Silurian also marks a period of dramatic increase of spores and megafossils that document the appearance and diversification of many important vascular plant groups (Kenrick and Crane, 1997a). These include the now extinct Protracheophytes and Rhyniopsids as well as lycopsid fossils in the Silurian-Devonian boundary (Kenrick and Crane, 1997b). The molecular divergence time estimate for tracheophytes (389.92 ± 64.71) is consistent with this fossil evidence. The Euphyllophytes (341.44 ± 58.59), Moniliformopses (263.36 ± 48.63) and seed plant clades (230.88 ± 43.85) show age estimates all slightly younger than the fossil record, Early, Middle, and Late Devonian, respectively (Kenrick and Crane, 1997a). Limited taxon sampling coupled with extinction should be considered when interpreting these results, as only a few exemplar taxa were used as placeholders for major lineages within land plants. Greater extant species (and molecular) diversity exists within the land plants than sampled here. Given this limitation, a remarkable level of consistency between the land-plant fossil record and the molecular divergence times was recovered.

The Charales divergence time was estimated at the Permian/Triassic boundary (247.74 ± 25.98 MYA) and this is supported by the occurrence of slightly younger *Aclistochara* (Characeae) from the Late Triassic (Liu and Chen, 1992). Similarities between living *Coleochaete* and Silurian-Devonian *Parka decipiens* have been proposed,

but their taxonomic placement remains unresolved (Graham, 1993; Hemsley, 1990; Niklas, 1976; Taylor and Taylor, 1993). *Parka* shares a discoid habit and corticated reproductive structures with some *Coleochaete* species, but ultrastructural differences in spore wall morphology suggest that *Parka* may be more closely related to modern liverworts (Graham, 1993; Hemsley, 1989). The molecular divergence times for *Coleochaete* and the liverwort lineage are nearly identical (460.12 ± 72.56 and 475.40 ± 81.09, respectively) with both being slightly older than the *Parka* fossil record. These dates unfortunately do not directly contribute to resolving the phylogenetic affinities of this enigmatic fossil.

Although there are no fossils unequivocally attributed to the Charales or land plants prior to the Ordovician, the Charales/land plant divergence time presented here (674 ± 99.69 MYA) might serve as a guide for fossil hunters to explore these sediments for land plant progenitors. Decay resistant polyphenolics and compounds similar to sporopollenin and lignin are found in living Zygnematales, Coleochaetales, Charales, and land plants (Delwiche et al., 1989; Graham, 1996; Kodner and Graham, 2001; Kroken et al., 1996). These compounds likely arose as an adaptive response to periods of desiccation, and given their distribution in extant taxa, such compounds might be found in Late Proterozoic rocks. Thus far, the fossil record has contributed little toward understanding the transition of green algae to the land, but further exploration of Late Proterozoic sediments may reveal insight into the early origin of the Charophyta and ultimately their descendants, the land plants.

Chapter IV: Phylogeny of *Nitella* (Charales: Charophyta)

Introduction

Overview

Commonly called stoneworts or brittleworts, the extant Charales are an evolutionarily important lineage not only in regard to their close relationship with embryophytes (Chapter II, Chapter III), but also with respect to their rich species diversity (Wood, 1965) and extensive fossil record (Feist and Grambast-Fessard, 1991; Grambast, 1974; Horn af Rantzien, 1959; Peck, 1953; Taylor et al., 1992). The Charales have attracted interest since the time of Plinius (1469) but were only later clearly diagnosed in the herbal of Bauhin (1623), as *Equisetum foetidum sub aqua repens* (i.e., an embryophyte). Vaillant (1721) assigned them to a new genus, *Chara*, which was later retained by Linnaeus (1753), who recognized four species. Currently the extant Charales contain one family, Characeae Ag., with two tribes and six genera; Chareae (Leonh.) Zanev. including *Chara* L., *Lamprothamnium* J. Groves, *Lychnothamnus* (Meyen) Leonh., and *Nitellopsis* Hy, and Nitelleae Gant. with *Nitella* Ag. em. A. Br., Leonh, and *Tolypella* (A. Br.) A. Br.

The Characeae often form a significant part of the submerged vegetation in slowly moving streams, ponds, and lakes throughout the world in all continents except Antarctica (Wood, 1965) and are commonly the first macrophytic vegetation to occupy new or recently inundated freshwater bodies (Crawford, 1977; Guerlesquin, 1991; Keiner, 1944; Wood, 1952). The Characeae often display complex species zonation in relation to depth and water clarity (Blindow, 1992; Corillion, 1957; de Winton et al.,

1991; Hutchinson, 1975; Schwarz et al., 2002; Spence and Crystal, 1970; Wood, 1950b). While typically found at depths less than 10 meters (Round, 1981), they have been reported growing as deep as 60 meters and are often the deepest macrophytic inhabitants of clear lakes (Dale, 1986; de Winton et al., 1991; Guerlesquin, 1991; Hutchinson, 1975; Starling et al., 1974; Vant et al., 1986). Some species, especially those in the genus *Lamprothamnium*, are found in brackish to hyper-saline waters, where they can by quite abundant (Corillion 1975, Brock 1981, Comin *et al*. 1993).

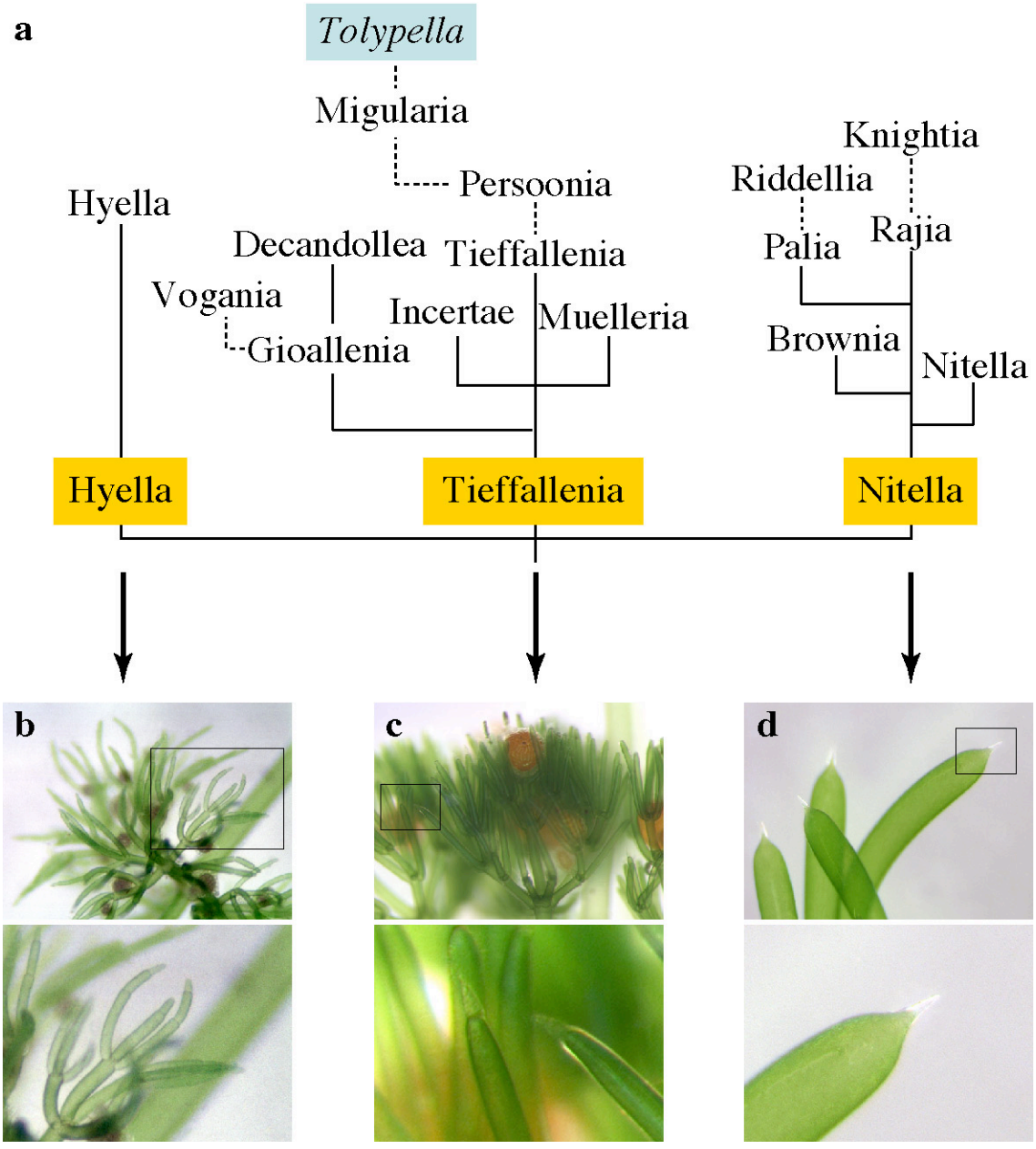
The genus *Nitella* is cosmopolitan and generally occurs in mildly acid or neutral freshwater environments (Corillion, 1957; Imahori, 1954; Olsen, 1944; Pal, 1932; Wood, 1965; Zaneveld, 1940). Fossils attributed to *Nitella* are represented from the Lower Cretaceous, Albian 112-99 MYA (Horn af Rantzien, 1957) and thalli with similar overall form date to the lower Devonian (*Palaeonitella*, 395-400 MYA) (Taylor et al., 1992). Modern *Nitella* exhibit a broad range of gross morphological diversity, from thalli with whorls of loosely arranged undifferentiated branchlets, to complex patterns of branchlet divisions (furcations), which can form dense tufts. Using scanning electron microscopy (SEM), intricate patterns found on the oospore membrane have been used to delineate species (Cáceres, 1975; Casanova, 1991; García, 1998; John and Moore, 1987; Leitch et al., 1990; Mandal and Ray, 1999; Mandal et al., 1995; Mukherjee and Ray, 1993; Nozaki et al., 1998; Ray et al., 2001; Sakayama et al., 2004; Sakayama et al., 2002). The monoecious or dioecious condition and the arrangement of gametangia (conjoined or sejoined) also have been extensively utilized in circumscribing species.

Given this morphological diversity it is not surprising that numerous taxonomic treatments have been proposed that date back to the older botanical literature (Allen,

1954; Braun and Nordstedt, 1882; Groves and Bullock-Webster, 1920; Halsted, 1879; Robinson, 1906; Wood, 1951; Wood, 1962; Zaneveld, 1940). The most recent and comprehensive treatment radically altered the classification of *Nitella* and of the Characeae in general (Wood, 1965). In this worldwide monograph, Wood (1965, p. 26) delimited taxa based on 'macroscopic and microscopic general morphology' rather than key characters (e.g., synapomorphies). As a result, he reduced the approximately 180 then recognized *Nitella* species to intra-specific ranks (i.e., variety or forma) or submerged them in synonymy yielding just nineteen broadly defined species. Wood (1965) argued that many species represented a continuum of morphological characters and suggested that 'genetically isolated' populations contributed to the complex pattern of morphological variation, but that this variation did not delineate true species. This concept has been challenged within the genus *Chara*. Data generated by numerous crossing experiments (McCracken et al., 1966; Proctor, 1970; Proctor, 1971; Proctor, 1972; Proctor, 1975; Proctor, 1980; Proctor et al., 1971; Proctor and Wiman, 1971) support greater species diversity than that proposed in Wood (1965).

The nineteen *Nitella* species recognized by Wood (1965) were divided into three subgenera (Nitella, Hyella, and Tieffallenia), and further separated into fifteen sections. The number and morphology of the cells following the last branchlet furcation, called a dactyl, were emphasized to define: (1) subgenus Nitella with single-celled dactyls, (2) subgenus Hyella with multi-celled (2-5) dactyls that terminate with allantoid-shaped cells, and (3) subgenus Tieffallenia, also with multi-celled dactyls (but generally restricted to two or sometimes three) that terminate with reduced, acute-shaped cells (Figure IV-1).

Figure IV-1. Hypothetical evolutionary sequence of *Nitella sensu* Wood (1965). **(1a)**, Evolutionary relationships among subgenera and sections of *Nitella* as hypothesized in Wood (1965) showing three subgenera (yellow boxes) and fifteen sections (black text). Note genus *Tolypella* (blue box) is tentatively placed within *Nitella* possibly derived from section Migularia. **(1b)**, *Nitella 'tasmanica'* KGK0078 (subgenus Hyella), showing female gametangia and multi-celled, allantoid-shaped dactyls (inset black box magnified below). **(1c)**, *Nitella hyalina* KGK0272 (subgenus Tieffallenia), showing both male and female gametangia and two-celled dactyls with reduced end cells (inset black box magnified below). Note that many of the end cells have fallen off giving the appearance of both single- and two-celled dactyls. **(1d)**, *Nitella clavata* KGK0232 (subgenus Nitella), showing single-celled dactyls with acuminate apex (inset black box magnified below).


a
Tolypella
Migularia
Persoonia
Hyella
Decandollea
Tieffallenia
Vogania
Incertae
Muelleria
Gioallenia
Knightia
Riddellia
Rajia
Palia
Brownia
Nitella
Hyella
Tieffallenia
Nitella
b
c
d

Wood (1965) relied on general vegetative morphology including dactyl morphology, patterns of branchlet furcation, and characteristics of the gametangia to delineate sections within *Nitella*. Though this classification is controversial, Wood (1965) brought together valuable morphological and distributional information and provided testable hypotheses toward understanding evolution, diversity and biogeography within this ancient group.

Several molecular-based phylogenetic studies have been published explicitly addressing generic relationships within the Characeae (McCourt et al., 1996a; McCourt et al., 1996b; Meiers et al., 1997; Sanders et al., 2003), and species relationships within *Chara* (McCourt et al., 1999; Meiers et al., 1999) and *Nitella* (Sakayama et al., 2004; Sakayama et al., 2002). Sakayama *et al*. (2002, 2004) investigated oospore membrane morphology using SEM and plastid encoded gene phylogenies to test the taxonomic scheme of Wood (1965). Their results suggested that the taxonomic system of Wood (1965) is unnatural, at least at the sectional level in subgenus Tieffallenia. Conclusions from these studies, however, are restricted by a limited number of *Nitella* species (19) and accessions from a limited geographic region (12 from Japan). To better understand diversity and evolution within *Nitella* worldwide, more extensive taxon sampling is necessary. Here a molecular phylogenetic analysis is presented using *rbcL* sequence data that more fully represents the worldwide diversity of *Nitella*.

Materials and Methods

DNA isolation, PCR, and sequencing

DNA was isolated from both living material and from dried herbarium specimens. Voucher material has been deposited in the University of Maryland Norton-Brown Herbarium (MARY). Epiphytes were removed under a Zeiss Stemi 2000-C dissecting microscope with tweezers or by brushing with a fine-haired paintbrush. To remove excess mucilage produced by some *Nitella* species, thalli were placed in glass Petri dishes containing 1% w:v N-acetyl-L-cysteine (Sigma A-7250) buffered with 2.0 mM HEPES pH 7.2 (Sigma H-7006) for ten minutes or until mucilage dissolved. After removing epiphytes and excess mucilage all living material was rinsed with a steady stream of de-ionized water, blotted with tissue to remove excess water, and placed in 1.7 ml centrifuge tubes at –20º C until time of extraction. Epiphytes were also removed from dried herbarium specimens when possible. Dried specimens, however, were often too delicate and brittle to manipulate extensively, therefore only the cleanest part of the herbarium specimen was selected and stored dry at room temperature until time of extraction.

DNA was extracted from frozen or dried thalli (0.01-0.1g) with the Nucleon Phytopure resin-based extraction kit using the protocol provided for small samples (Amersham Pharmacia, Uppsala, Sweden) with one modification. Rather than grinding tissue in liquid nitrogen, frozen or dried tissue was ground directly in 200 µl 'Reagent 1' until homogeneous, after which an additional 400 µl was added. The remainder of the manufacturer's protocol was followed without modification. Quality of the extracted DNA was assessed via agarose gel electrophoresis and ethidium bromide staining.

Amplification of *rbcL* was performed in two ways. Initially, the polymerase chain reaction (PCR) was attempted on all samples with primers RH1 [ATGTCACCACAAACAGAAACTAAAGC: (Zurawski and Clegg, 1987) and 1385R [AATTCAAATTTAATTTCTTTCC; (Manhart, 1994)]. Often DNA isolated from dried herbarium material did not amplify with the RH1-1385R primer pair and two individual reactions were required, each amplifying a smaller fragment of the gene: (1) with primer RH1 and Characeae specific primer *rbcL*-972R (ATCACCACCAGAAAGACGAAG) and (2) Characeae specific primer *rbcL*-295F (GCATATGTTGCTTATCCTCTT) and 1385R. These reactions yielded fragments which over lapped by 638 base pairs (bp), excluding primers. The resulting PCR products were purified using a modified polyethylene glycol (PEG) precipitation (Morgan and Soltis, 1993). An equal volume of 20% w:v PEG 8000, 2.5 M NaCl was added to each PCR product, vortexed briefly and spun at 16,000g for 15 minutes. The solution was removed and the resulting DNA pellet was washed once with 70% cold ethanol. After removing the ethanol, the pellet was air-dried and resuspended in 25 µl de-ionized water. The PEG-purified PCR product was quantified via agarose gel electrophoresis and ethidium bromide staining for subsequent sequencing reactions.

Sequencing reactions were performed in 7 µl final volume (3.0 µl PEG-purified PCR product, 0.7 µl de-ionized water, 1.3 µl 2.5 µM primer, 1.5 µl 5X buffer [400 mM Tris pH 9.0, 10 mM $MgCl_2$], 0.5 µl BigDye Terminator Ready Reaction Mix v2 [Perkin Elmer Biosystems, Foster City, CA]), cycled and purified according to the manufacturer's protocols and resolved using either an ABI 377 slab gel sequencer (performed by the University of Maryland Center for Agricultural Biotechnology) or an

ABI 3100 capillary sequencer. Sequences were determined initially on single strand reads using primers RH1 and 1385R. The resulting sequences were edited and compiled with the computer program Sequencher 3.1.1 (Gene Codes Corporation, Ann Arbor, MI). If sequences from multiple isolates of the same species were found to be identical, one individual was arbitrarily chosen for further sequencing. All unique sequences including an individual representative of multiple isolates of the same species were then determined on the complimentary strand with primers *rbcL*-670F (GCAATTTATAAATCTCAAGCAG) and *rbcL*-751R (GCATTTCTTCACAAGTTCCTG).

Newly determined sequences were combined with sequences from the literature. Twenty *rbcL* sequences representing twelve species of *Nitella* have been published previously (one sequence, Manhart, 1994; three sequences for three species, McCourt *et al*., 1999; twenty eight sequences for sixteen species, Sakayama *et al*., 2002; 2004). The sequence from Manhart (1994) was identified as *N. translucens* (Genbank accession number L13482); however, this sequence showed strong affinity with *N. axillaris* and not *N. translucens* (results not shown). Because a voucher for this specimen could not be found to confirm identification, L13482 was not included in this study. Sequences from Sakayama *et al*. (2002; 2004) were shorter than those presented here (1194 bp *versus* 1353 bp, respectively), therefore, identical sequences determined for this study from the same species were included in place of the shorter sequences. Outgroup taxa were selected to represent the diversity of embryophytes and include all published Coleochaetales *rbcL* sequences. To facilitate comparison with other studies, embryophyte taxa were selected to match those used in Chapter III and Coleochaetales

sequences were derived from Turmel *et al*. (2002a), Delwiche *et al*. (2002) and Cimino and Delwiche (2002). All sequences were aligned by hand and no insertion/deletion events or internal stop codons were detected. A summary of species and source information is shown in Appendix II.

Phylogenetic analyses

Phylogenetic analyses were performed using two computer programs: (1) PAUP* 4.0b10 (Swofford, 2002) was used for maximum parsimony (MP) and maximum likelihood (ML) analyses, and (2) MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) was used for Bayesian inference (BI). For MP analyses characters were unordered and assigned equal weights at all sites (Fitch, 1971). Ten heuristic search replicates were performed with random-taxon-addition, tree bisection reconnection (TBR) branch swapping, steepest decent and 'MulTrees' options in effect. For ML and BI analyses, Modeltest 3.06 (Posada and Crandall, 1998) was used to select among 56 nested models of sequence evolution. Under the Akaike information criterion (AIC), the best fitting model was found to be the general-time-reversible model with invariant sites and gamma distributed rates for variable sites (GTR+I+Γ). A ML heuristic search using this model and parameters values estimated by Modeltest was performed with a single random taxon addition sequence, nearest-neighbor-interchange (NNI) branch swapping and steepest descent option active. Parameters were re-estimated using the resulting tree, and three iterative searches were performed until they converged on similar values. These values were then fixed and used for a ML analysis with three heuristic search replicates, random-taxon-addition, tree-bisection-reconnection (TBR) branch swapping, steepest decent and 'MulTrees' options in effect.

One hundred bootstrap pseudoreplicates were generated using CodonBootstrap 3.0b4 (Bollback, 2001), taking into account protein-coding portions (codons) of the dataset, and used for MP and ML bootstrap analyses (Felsenstein, 1985). MP bootstrap replicates were each performed with three random-taxon-addition TBR heuristic searches and ML bootstrap replicates were performed each with three random-taxon-addition NNI heuristic searches using parameter values described above.

MrBayes uses a Metropolis-coupled Markov chain Monte Carlo (or MCMCMC) algorithm to generate an approximation of the posterior probability distribution of phylogenies. Two models implemented in MrBayes were examined: (1) GTR+I+Γ for comparison with the ML analysis, and (2) GTR+I+Γ with 'covarion' option active. The covarion option allows the rate at a site to change at any given time over its evolutionary history thereby allowing the pool of variable sites (I) to change across the tree. For each BI model, four separate runs were carried out each with four Markov chains starting from a random tree. Three of these chains were heated allowing for broad sampling of parameter space. Each run was allowed to continue for two million generations sampling every 100 generations for a total of 20,000 samples per run. The tree scores were plotted against the corresponding generation number to assess that the chain had reached stationarity. In this way, generations sampled before the chain reached stationarity were identified and discarded as burn-in. Independent runs using the same model of sequence evolution were then compared and if essentially identical tree topologies and posterior probabilities were found, indicating convergence and appropriate mixing, the tree files and parameter files were combined and summarized using MrBayes 'sumt' and 'sump' commands.

Results and Discussion

Taxa and data set

This study presents *rbcL* sequence data for 177 operational taxonomic units (OTUs) with a total length of 1,353 base pairs. Eighty OTUs represented identical *Nitella* sequences from multiple isolates of the same species and were not included in phylogenetic analyses. The remaining 100 representatives of Characeae included seven *Chara*, three *Lamprothamnium*, three *Lychnothamnus*, two *Nitellopsis*, six *Tolypella* and seventy-nine unique *Nitella* sequences. Eight land plant and twelve Coleochaetales sequences were used as out groups. A character-base summary for each major lineage is presented in Table IV-1.

Table IV-1. Mean base composition values across taxa, parsimony informative characters (PIC), parsimony uninformative characters (PUC), constant characters (CC), total characters (Total), and number of operational taxonomic units used in phylogenetic analyses (OTU) for *Nitella*, Characeae, Coleochaetales, land plants, and all taxa respectively.

	%A	%C	%G	%T	PIC	PUC	CC	Total	OTU
Nitella	30	15	23	32	270	53	1030	1353	79
Characeae	30	15	23	32	355	43	955	1353	100
Coleochaetales	31	16	21	32	243	87	1023	1353	12
Land plants	27	19	24	30	270	213	870	1353	8
All taxa	30	16	22	32	530	80	743	1353	120

All phylogenetic analyses resulted in essentially identical tree topologies with differences associated with weakly supported nodes. The MP analyses yielded eight most parsimonious trees 2,767 steps in length, with a consistency index (CI) of 0.3488 and a retention index (RI) of 0.7927; ML analyses resulted in a single tree (–ln = 15459.479). The BI tree using GTR+I+Γ with the covarion option active is shown in Figure IV-2. The monophyly of the Characeae was strongly supported in all analyses as was monophyly of the tribe Chareae (Figure IV-3). Unlike results presented in Chapter III, *Chara* formed a monophyletic group sister to a strongly supported *Lamprothamnium* clade, although support for *Chara* was relatively weak. Similar to Chapter III, the tribe Nitelleae was paraphyletic, with *Tolypella* sister to a clade composed of the tribe Chareae + *Nitella*. The monophyly of *Nitella* showed mixed levels of support depending on analytical method (i.e., both Bayesian analyses recovered higher support values than the ML or MP analyses). The clade containing Chareae + *Nitella* was weakly supported under all methods suggesting the need for further data to resolve these relationships.

Within *Nitella* two distinct basal lineages were recovered (Figure IV-3). One lineage includes part of subgenus Nitella, and is comprised of the '*N. flexilis* group' (*Nitella flexilis*, *N. mirabilis, N. missouriensis*, and *N. opaca*) and the '*N. stuartii* group' (*N. macounii*, and *N. stuartii*). The other lineage includes the remaining members of subgenus Nitella sampled in this study, termed the '*N. acuminata* group' (including *N. acuminata*, *N. bastinii*, *N. clavata*, and *N. praelonga*), and all remaining members of the genus [subgenera Tieffallenia and Hyella (Figure IV-4, 5)]. Paraphyly of subgenus Nitella was also identified in a recent phylogenetic analysis using *rbcL* and *atpB* sequence data with a limited number of taxa (Sakayama et al., 2004).

Morphological characters have not been identified that clearly separate the *N. flexilis*/*N. stuartii* lineage from the remainder of *Nitella*, however, chromosome number may be a useful synapomorphy marking this basal split. The base chromosome number for *Nitella* is likely x = 3, or possibly x = 6 (Guerlesquin, 1967) and monoecious species of the Characeae generally have twice the chromosome number as their dioecious counterparts (Gillet, 1959; Guerlesquin, 1967; Hotchkiss, 1963; Imahori and Kato, 1961; Tindall, 1967: Tindall and Sawa, 1964). Dioecious members if the '*N. flexilis* group' (*N. missouriensis*, *N. opaca*, *N. mirabilis*) have chromosome number *n* = 6, while monoecious species (*N. flexilis*) exibit *n* = 12 chromosomes. An accurate chromosome count of *N. macounii* has been elusive (Mann and Raju, 2002) and *n* = 15 has been reported for *N. stuartii* (Guerlesquin, 1967; Michelle Casanova, personal communication), possibly representing either a duplication or reduction of the base number of three chromosomes (from twelve or eighteen to fifteen). The remaining members of subgenus Nitella sampled in this study (*N. acuminata*, *N. bastinii*, *N. clavata*, and *N. praelonga*) as well as subgenera Tieffallenia and Hyella generally have dioecious species with chromosome number *n* = 9 and monoecious species with chromosome number *n* = 18. In general, the basal split in *Nitella* corresponds nicely with published chromosome numbers of *n* = 6/12 (15) for a select few members of subgenus Nitella [the '*n* = 6/12 (15)' clade], and *n* = 9/18 for the remaining members of this genus (the '*n* = 9/18' clade).

FIGURE IV-2. Phylogenetic relationships for the Charales determined by Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses using *rbcL* sequence data. Values in parentheses adjacent to taxon names represent the total number of individuals sampled with that name and identical *rbcL* sequence. Values not in parentheses represent the clone number associated with that unique sequence, and species without numbers were represented by a single sequence (Appendix II). BI posterior probabilities using the GTR+I+Γ with the covarion option active, and GTR+I+Γ (covarion option not active) are presented above branches, respectively. ML and MP bootstrap values for 100 replicates are presented below branches, respectively. Support values are only shown for the Coleochaetales, land plants, and Charales and support values within the Charales are shown in Figure IV-2 to IV-4. The Coleochaetales, land plants, Chareae, and Nitelleae (*Nitella* and *Tolypella*) are shown with brackets on the right. The tree is rooted with the Coleochaetales and branch lengths are Bayesian mean values from analyses using GTR+I+Γ with the covarion option active and are proportional to the number of substitutions per sites.

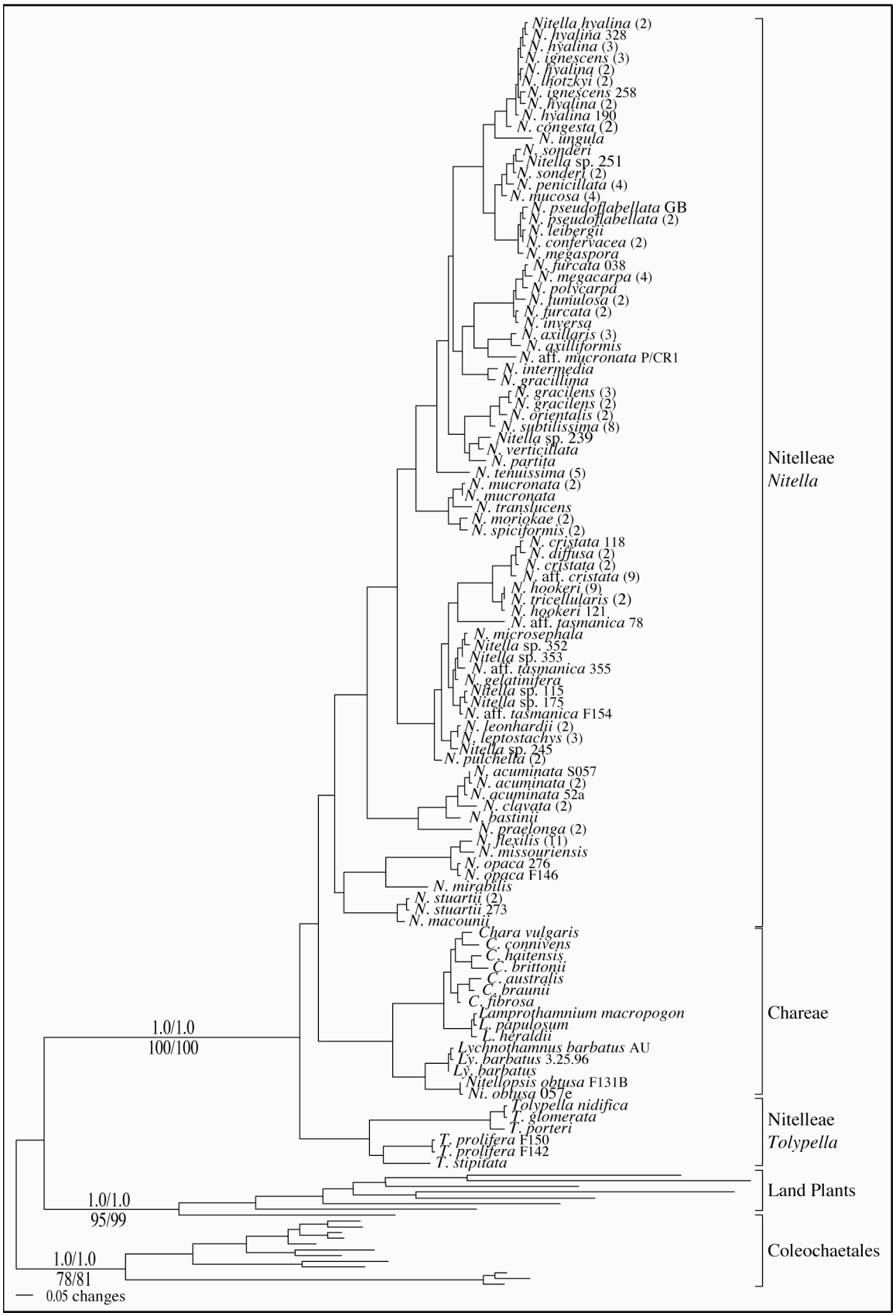
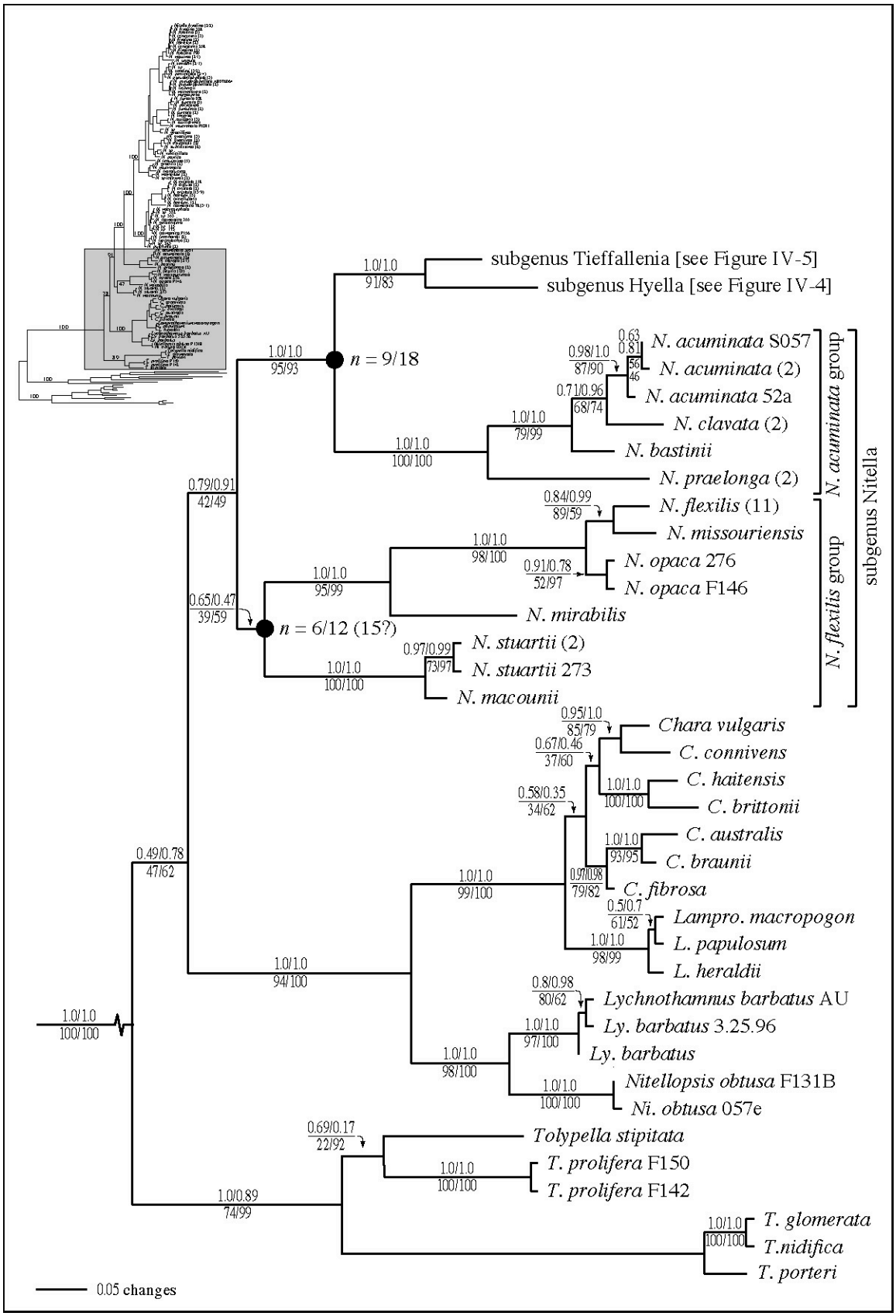

Nitella hyalina (2)
N. hyalina 328
N. hyalina (3)
N. ignescens (3)
N. hyalina (2)
N. hiotzkyi (2)
N. ignescens 258
N. hyalina (2)
N. hyalina 190
N. congesta (2)
N. ungula
N. sonderi
Nitella sp. 251
N. sonderi (2)
N. penicillata (4)
N. mucosa (4)
N. pseudoflabellata GB
N. pseudoflabellata (2)
N. leibergii
N. confervacea (2)
N. megaspora
N. furcata 038
N. megacarpa (4)
N. polycarpa
N. tumulosa (2)
N. furcata (2)
N. inversa
N. axillaris (3)
N. axilliformis
N. aff. mucronata P/CR1
N. intermedia
N. gracillima
N. gracilens (3)
N. gracilens (2)
N. orientalis (2)
N. subtilissima (8)
Nitella sp. 239
N. verticillata
N. partita
N. tenuissima (5)
N. mucronata (2)
N. mucronata
N. translucens
N. moriokae (2)
N. spiciformis (2)
N. cristata 118
N. diffusa (2)
N. cristata (2)
N. aff. cristata (9)
N. hookeri (9)
N. tricellularis (2)
N. hookeri 121
N. aff. tasmanica 78
N. microsephala
Nitella sp. 352
Nitella sp. 353
N. aff. tasmanica 355
N. gelatinifera
Nitella sp. 115
Nitella sp. 175
N. aff. tasmanica F154
N. leonhardii (2)
N. leptostachys (3)
Nitella sp. 245
N. pulchella (2)
N. acuminata S057
N. acuminata (2)
N. acuminata 52a
N. clavata (2)
N. bastinii
N. praelonga (2)
N. flexilis (11)
N. missouriensis
N. opaca 276
N. opaca F146
N. mirabilis
N. stuartii (2)
N. stuartii 273
N. macounii
Chara vulgaris
C. connivens
C. haitensis
C. brittonii
C. australis
C. braunii
C. fibrosa
Lamprothamnium macropogon
L. papulosum
L. heraldii
Lychnothamnus barbatus AU
Ly. barbatus 3.25.96
Ly. barbatus
Nitellopsis obtusa F131B
Ni. obtusa 057e
Tolypella nidifica
T. glomerata
T. porteri
T. prolifera F150
T. prolifera F142
T. stipitata
1.0/1.0
100/100
1.0/1.0
95/99
1.0/1.0
78/81
0.05 changes
Nitelleae
Nitella
Chareae
Nitelleae
Tolypella
Land Plants
Coleochaetales

FIGURE IV-3. Phylogenetic relationships for basal lineages of *Nitella* subgenus Nitella *sensu* Wood (1965) determined by Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses using *rbcL* sequence data. Values in parentheses adjacent to taxon names represent the total number of individuals sampled with that name and identical *rbcL* sequence. Values not in parentheses represent the clone number associated with that unique sequence, and species without numbers were represented by a single sequence (Appendix II). Black dots at nodes show inferred ancestral chromosome number. BI posterior probabilities using the GTR+I+Γ with the covarion option active, and GTR+I+Γ (covarion option not active) are presented above branches, respectively. ML and MP bootstrap values for 100 replicates are presented below branches, respectively. The top left inset is identical to Figure IV-2 and the shaded box shows the magnified area of the tree. Branch lengths are Bayesian mean values from analyses using GTR+I+Γ with the covarion option active and are proportional to the number of substitutions per sites.


subgenus Tieffallenia [see Figure IV-5]
subgenus Hyella [see Figure IV-4]
n = 9/18
n = 6/12 (15?)
N. acuminata S057
N. acuminata (2)
N. acuminata 52a
N. clavata (2)
N. bastinii
N. praelonga (2)
N. flexilis (11)
N. missouriensis
N. opaca 276
N. opaca F146
N. mirabilis
N. stuartii (2)
N. stuartii 273
N. macounii
N. acuminata group
N. flexilis group
subgenus Nitella
Chara vulgaris
C. connivens
C. haitensis
C. brittonii
C. australis
C. braunii
C. fibrosa
Lampro. macropogon
L. papulosum
L. heraldii
Lychnothamnus barbatus AU
Ly. barbatus 3.25.96
Ly. barbatus
Nitellopsis obtusa F131B
Ni. obtusa 057e
Tolypella stipitata
T. prolifera F150
T. prolifera F142
T. glomerata
T.nidifica
T. porteri
0.05 changes

The 'n = *6/12 (15)' clade*

Sectional relationships (*sensu* Wood) within the '*n* = 6/12 (15)' clade were difficult to reconcile with the *rbcL* phylogeny. According to Wood (1965), section Nitella includes three species (*Nitella flexilis*, *N. mirabilis*, and *N. macounii*), several varieties, and numerous formas. The eleven isolates of monoecious *N. flexilis*, from Germany, Japan, and the United States, shared identical *rbcL* sequences and were found sister to dioecious *N. missouriensis* from southwestern U.S. (Figure IV-3). The placement of *N. missouriensis* close to *N. flexilis* is contrary to Wood (1965), who placed *N. missouriensis* in synonymy with *N. acuminata* in section Rajia ('*n* = 9/18' clade). Two isolates of dioecious *N. opaca*, one from Poland and a first report from New Zealand, were resolved sister to the *N. flexilis*/*N. missouriensis* clade (Figure IV-3). The most apparent morphological character uniting *N. flexilis*, *N. missouriensis*, *N. mirabilis*, and *N. opaca* is the distinctive oogonial jacket cells that are apically swollen at maturity (Wood, 1965).

Wood (1965) did not consider sexual state to be an important criterion for species delineation and *N. flexilis* and *N. opaca* are not easily distinguished if fertile material is unavailable (or ignored); consequently Wood (1965) placed *N. opaca* in synonomy with *N. flexilis*. Detailed cytological evidence of both *N. flexilis* and *N. opaca* from the U.S. supported *N. opaca* as a distinct species separate from *N. flexilis* and further supported the utility of sexual state as a species-level character, at least for this complex (Sawa, 1965).

Oospore membrane ornamentation also reveals a close relationship of the '*N. flexilis* group.' Horn af Rantzein (1959) recognized the distinctive nature of *Nitella flexilis* when he used LM to describe the oospore membrane as 'minutely and very obscurely granulate, faveolate or smooth.' Subsequent SEM studies have revealed a smooth to spongy oospore surface for *N. flexilis* and *N. opaca* (Frame, 1977; Leitch et al., 1990). Sakayama *et al*. (2004) described the oospore surface of *N. mirabilis* from Japan as finely granulate under LM, but demonstrated that a network of fibrils forming a sponge-like network was present under SEM. A granulate oospore membrane has been described for *N. missouriensis* using LM (Allen, 1954), however conformation using SEM is needed. The phylogenetic position of *N. opaca* sister to the *N. flexilis*/*N. missouriensis* clade (not imbedded among the eleven *N. flexilis* isolates) adds further evidence for the species-level status of this taxon (Figure IV-3).

Mann and Raju (2002) reported sixteen new localities for the rare ephemeral *Nitella macounii* (T. F. Allen) T. F. Allen and clarified several poorly understood morphological characters, including dactyl number and shape, oospore ornamentation, and antheridia morphology. *Nitella macounii* exhibited single-celled dactyls with both acute and acuminate apices (Mann and Raju, 2002), contrary to reports of multicelled dactyls (Allen, 1888; Wood, 1965; Wood and Imahori, 1964). The multicelled condition may have been a misinterpretation of incomplete abscission and/or suppressed furcation producing the appearance of multicelled dactyls (Allen, 1954; Mann and Raju, 2002). The oospore membrane surface under SEM showed a range of morphological variation from reticulate to tuberculate (Crum, 1975; Frame, 1977; Mann and Raju, 2002). This variation appeared to be correlated with oospore color and possibly maturity; lighter and

presumably younger oospores were incompletely reticulate while the darkest/most mature oospores were generally tuberculate (Mann and Raju, 2002). Finally, antheridia of *N. macounii* were composed of four shield cells (scutes), rather than the common condition for the Characeae of eight. Only a few *Nitella* species (*N. stuartii* A. Br., *N. terrestris* Iyengar, *N. quadriscutulum* Jao and Li, and *N. cordobensis* Cáceres) and one *Chara* species (*Chara zeylanica* Klein ex Willd.) have antheridia composed of four shield cells and this has been show to be taxonomically significant in clarification of the *Chara zeylanica* species complex (Proctor et al., 1971).

Owing to inconsistent morphological interpretations and incomplete material, it is not surprising that several taxonomic placements have been proposed for *N. macounii*. T. F. Allen (1888) originally suggested a close affinity with *N. stuartii* from New Zealand. Wood (1965) included *N. macounii* in section Nitella with *N. flexilis* and *N. mirabilis*. Tindall (1967) suggested that *N. macounii* belonged in a group including *N. acuminata*, *N. stuartii*, and *N. allenii*. The phylogenetic analyses presented here support *N. macounii* sister to *N. stuartii* (section Palia), represented by two isolates from Australia and one from New Zealand, and not closely related to *N. acuminata* (Figure IV-3). *Nitella macounii* shares several morphological features with *N. stuartii* including heteroclemous branchlets (advantageous branchlets found in addition to the primary whorl of branchlets), though *N. macounii* has been interpreted as homoclemous (Allen, 1954), or exhibiting only a 'heteroclemous tendency' (Mann and Raju, 2002). These species also share two- or sometimes three-furcate branchlets with single-celled dactyls, and most notably tetrascutate antheridia.

Wood (1965) considered subgenus Nitella to be a monophyletic group united by single-celled dactyls (Figure IV-1). The *rbcL* results suggest that single-celled dactyls may represent a pliesiomorphic condition for *Nitella* (Figure IV-3) and chromosome number, though not always practical, may be a more reliable character to distinguish natural, basal lineages in *Nitella*.

FIGURE IV-4. Phylogenetic relationships for *Nitella* subgenus Hyella (*sensu* Wood 1965) determined by Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses using *rbcL* sequence data. Values in parentheses adjacent to taxon names represent the total number of individuals sampled with that name and identical *rbcL* sequence. Values not in parentheses represent the clone number associated with that unique sequence, and species without numbers were represented by a single sequence (Appendix II). BI posterior probabilities using the GTR+I+Γ with the covarion option active, and GTR+I+Γ (covarion option not active) are presented above branches, respectively. ML and MP bootstrap values for 100 replicates are presented below branches, respectively. The top left inset is identical to Figure IV-2 and the shaded box shows the magnified area of the tree. Branch lengths are Bayesian mean values from analyses using GTR+I+Γ with the covarion option active and are proportional to the number of substitutions per sites.

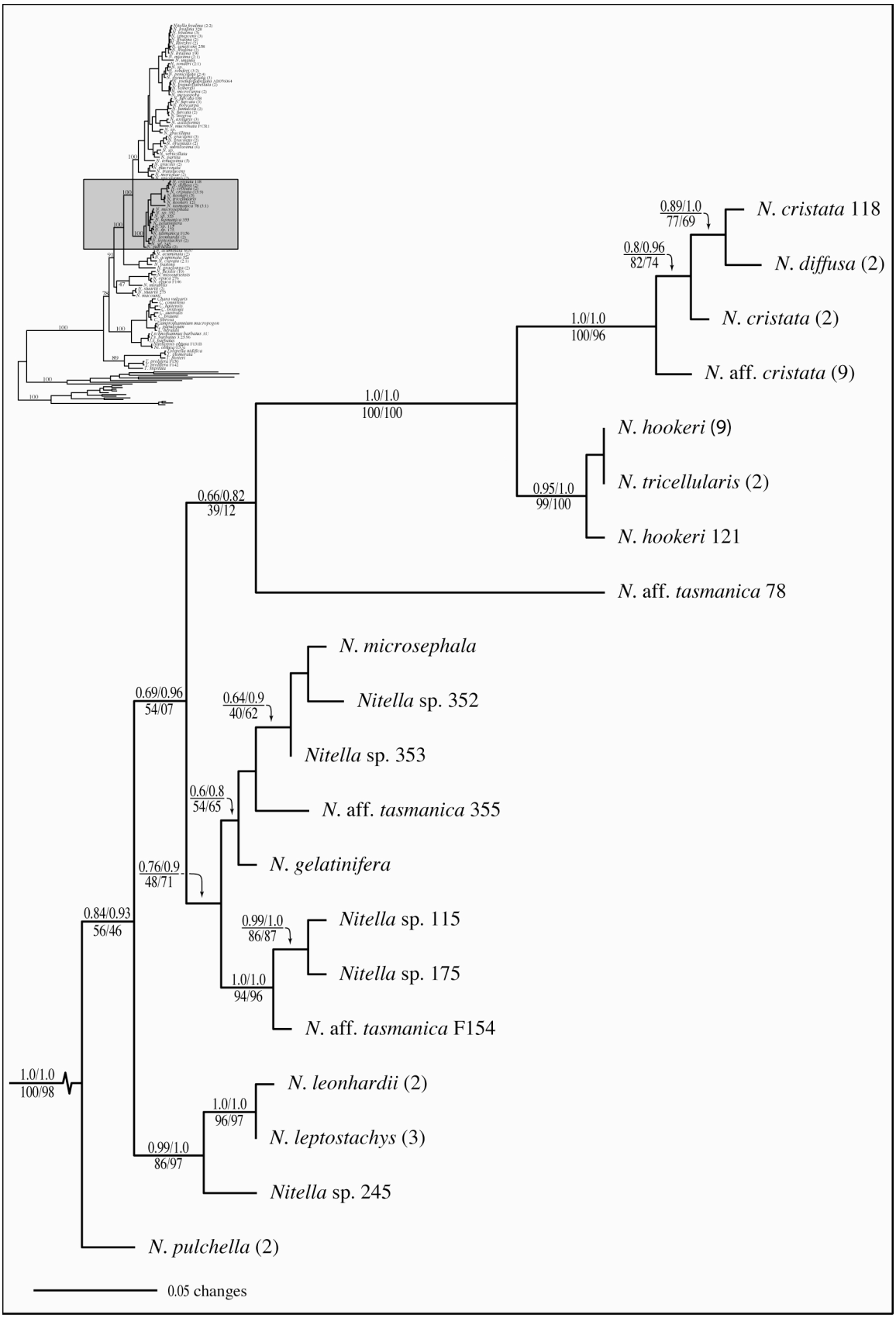
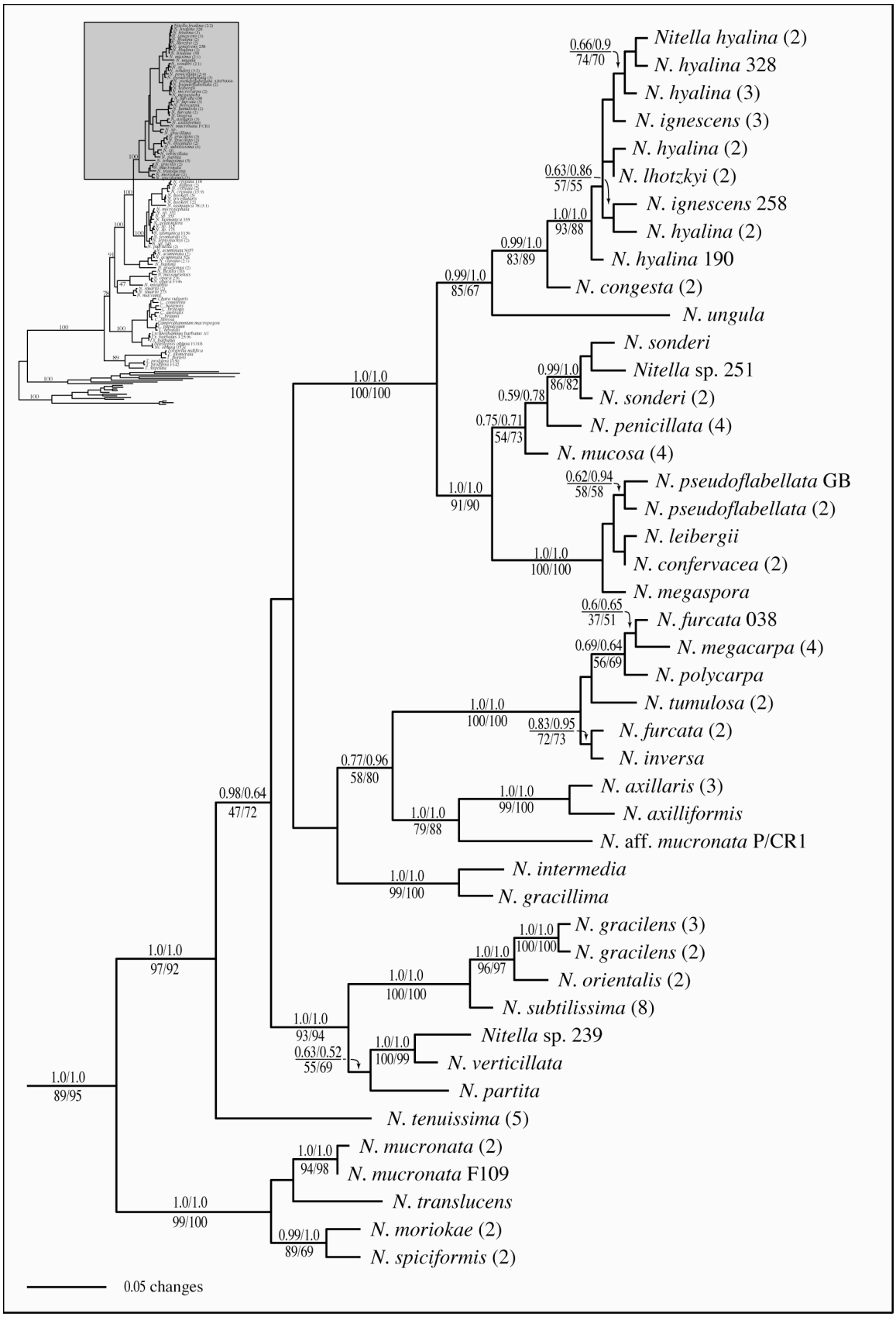

0.89/1.0
77/69
N. cristata 118
0.8/0.96
82/74
N. diffusa (2)
1.0/1.0
100/96
N. cristata (2)
N. aff. cristata (9)
1.0/1.0
100/100
N. hookeri (9)
N. tricellularis (2)
0.95/1.0
99/100
N. hookeri 121
0.66/0.82
39/12
N. aff. tasmanica 78
N. microsephala
0.69/0.96
54/07
0.64/0.9
40/62
Nitella sp. 352
Nitella sp. 353
0.6/0.8
54/65
N. aff. tasmanica 355
N. gelatinifera
0.76/0.9
48/71
0.84/0.93
56/46
0.99/1.0
86/87
Nitella sp. 115
Nitella sp. 175
1.0/1.0
94/96
N. aff. tasmanica F154
1.0/1.0
100/98
N. leonhardii (2)
1.0/1.0
96/97
N. leptostachys (3)
0.99/1.0
86/97
Nitella sp. 245
N. pulchella (2)
0.05 changes

FIGURE IV-5. Phylogenetic relationships for *Nitella* subgenus Tieffallenia *sensu* Wood (1965) determined by Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses using *rbcL* sequence data. Values in parentheses adjacent to taxon names represent the total number of individuals sampled with that name and identical *rbcL* sequence. Values not in parentheses represent the clone number associated with that unique sequence, and species without numbers were represented by a single sequence (Appendix II). BI posterior probabilities using the GTR+I+Γ with the covarion option active, and GTR+I+Γ (covarion option not active) are presented above branches, respectively. ML and MP bootstrap values for 100 replicates are presented below branches, respectively. The top left inset is identical to Figure IV-2 and the shaded box shows the magnified area of the tree. Branch lengths are Bayesian mean values from analyses using GTR+I+Γ with the covarion option active and are proportional to the number of substitutions per sites.


0.66/0.9
74/70
Nitella hyalina (2)
N. hyalina 328
N. hyalina (3)
N. ignescens (3)
N. hyalina (2)
0.63/0.86
57/55
N. lhotzkyi (2)
N. ignescens 258
1.0/1.0
93/88
N. hyalina (2)
0.99/1.0
83/89
N. hyalina 190
0.99/1.0
85/67
N. congesta (2)
N. ungula
N. sonderi
Nitella sp. 251
0.99/1.0
86/82
N. sonderi (2)
0.59/0.78
N. penicillata (4)
0.75/0.71
54/73
N. mucosa (4)
1.0/1.0
100/100
1.0/1.0
91/90
0.62/0.94
58/58
N. pseudoflabellata GB
N. pseudoflabellata (2)
N. leibergii
1.0/1.0
100/100
N. confervacea (2)
N. megaspora
0.6/0.65
37/51
N. furcata 038
0.69/0.64
56/69
N. megacarpa (4)
N. polycarpa
N. tumulosa (2)
1.0/1.0
100/100
0.83/0.95
72/73
N. furcata (2)
N. inversa
0.77/0.96
58/80
1.0/1.0
99/100
N. axillaris (3)
1.0/1.0
79/88
N. axilliformis
N. aff. mucronata P/CR1
1.0/1.0
99/100
N. intermedia
N. gracillima
0.98/0.64
47/72
1.0/1.0
100/100
N. gracilens (3)
1.0/1.0
96/97
N. gracilens (2)
1.0/1.0
100/100
N. orientalis (2)
N. subtilissima (8)
1.0/1.0
97/92
1.0/1.0
93/94
Nitella sp. 239
1.0/1.0
100/99
0.63/0.52
55/69
N. verticillata
N. partita
1.0/1.0
89/95
N. tenuissima (5)
1.0/1.0
94/98
N. mucronata (2)
N. mucronata F109
N. translucens
1.0/1.0
99/100
0.99/1.0
89/69
N. moriokae (2)
N. spiciformis (2)
0.05 changes

The 'n = *9/18' clade*

Within the '*n = 9/18'* clade two distinct subclades were recovered, one comprised of species with single-celled dactyls (*N. acuminata* A. Br. ex Wallm. , *N. bastinii* T. F. Allen, *N. clavata* Kütz, and *N. praelonga* A. Br.) and the other of species with multicelled dactyls (Figure IV-3). This latter clade is further divided into a group with multicelled dactyls (≥ 3) roughly corresponding with subgenus Hyella (Figure IV-4) and a group with just two-celled (or sometime two- and three-celled) dactyls corresponding with subgenus Tieffallenia (Figure IV-5).

The single-celled dactyl group includes representatives from three sections *sensu* Wood (1965); Brownia (*Nitella clavata*), Rajia (*N. acuminata* and *N. bastinii*), and Riddellia (*Nitella praelonga*). All members of this clade share acuminate dactyl apices and coronal jacket cells that are not apically swollen at maturity. The two *N. praelonga* isolates, one from Tamaulipas, Mexico, and the other from the Panamanian/Costa Rican border, shared identical *rbcL* sequences and were found sister to a paraphyletic Rajia, which included the only representative of section Brownia (*N. clavata*) (Figure IV-3). *Nitella bastinii* was thought to be unknown since its discovery on the grounds of the 1890 Chicago World Fair (Wood, 1965), however, it has subsequently been reported from Iowa by Crum (1975), and discovered to be wide spread in Llano Estacado (High Plains of the Texas Panhandle and adjacent New Mexico) (Proctor, 1990). The *rbcL* data placed the single representative of *N. bastinii* from Texas, sister to a clade including *N. clavata* and several isolates of *N. acuminata*.

Four isolates of *N. acuminata* were sampled, one from Japan (Sakayama et al., 2004), two collected by Vernon Proctor from different localities in Costa Rica, and a fourth from northern Wisconsin. The isolates from Costa Rica and Wisconsin exhibited axillary and rarely terminal reduced fertile heads corresponding to *N. acuminata* var. *greenii* R.D.W., and axillary fertile heads were reported lacking from the Japanese isolate (Sakayama et al., 2004), consistent with *N. acuminata* var. *acuminata* f. *acuminata* [*sensu* Wood (1965)]. The *rbcL* phylogeny resolved the two isolates from Costa Rica sister to the Japanese isolate. The Wisconsin isolate was found sister to these. Support within the *N. acuminata* clade was relatively weak, but monophyly of the species complex was strongly supported (Figure IV-3). The *N. acuminata* group includes numerous described 'species' exhibiting considerable morphological variation and Wood (1965) emphasized dactyl size (reduced or elongate) and the presence of axillary fertile heads to circumscribe subspecific groups in this taxon. Observations of cultured material (Tindall, 1970) demonstrated that light and temperature were key factors in development of these characters, and suggested that species collected from southwestern United States and northern Mexico were conspecific. Interestingly, all of the samples studied by Tindall (1970) were monoecious and had chromosome number $n = 9$, not $n = 18$ as previously reported for the 'same' monoecious species from northern United States (Hotchkiss, 1958; Wood, 1954), Japan (Imahori and Kato, 1961; Sasaki, 1961), India (Sarma and Khan, 1964), and Fiji (Hotchkiss, 1965). Oospore ornamentation for the $n = 9$ isolates was described as finely granulate under LM (Tindall, 1970); however, SEM revealed a scabrous oospore surface composed of irregularly arranged projections for *N. acuminata* var. *greenii* (Leitch et al., 1990) and *N. acuminata* var. *acuminata* (Sakayama et al.,

2004). Detailed analyses of species in the *N. acuminata* group using a combination of chromosome counts, SEM oospore ornamentation, and molecular phylogentic methods are needed to elucidate the taxonomy and evolutionary history of this species complex.

Subgenus Hyella

Figure IV-4 presents the *rbcL* phylogeny for all members of *Nitella* that share equal to or greater than three cells per dactyl. Species in this clade generally correspond with those of subgenus Hyella R.D.W. with two major exceptions. Despite having multicelled dactyls, Wood (1965) emphasized the conical shape of the dactyl apices and included *Nitella cristata* A. Br. em. in subgenus Tieffallenia, section Migularia. *Nitella hookeri* A. Br. was grouped in subgenus Nitella, section 'Incertae' with single-celled dactyl species because sufficient material was not available to arrive at a satisfactory revision of this species (Wood, 1965). Subsequent examinations of *N. hookeri* material led Wood and Mason (1977) to dismantle section 'Incertae' and place *N. hookeri* in section Migularia along with *N. cristata* in subgenus Tieffallenia. The *rbcL* data placed *N. hookeri*, *N. hookeri* var. *tricellularis* Nordst., *N. cristata*, and *N. cristata* f. *diffusa* (A. Br.) R.D.W. with other multicelled-dactyl species in subgenus Hyella. Wood emphasized dactyl end-cell shape (conical vs. allantoid) rather than dactyl cell number (two vs. numerous) when placing these species in Tieffallenia, but data presented here support the inclusion of all taxa with multicelled dactyls in a single, strongly supported, monophyletic group (Figure IV-4).

Nitella hookeri and *N. hookeri* var. *tricellularis* cannot be differentiated with the *rbcL* data alone; however, branchlet morphology can be used to distinguish between

these two taxa. Plants with clearly furcate sterile branchlets, and slightly compacted fertile branchlets that do not form dense heads characterize *N. hookeri*. Plants with simple or brachydactylous sterile branchlets, and compact fertile heads, characterize *N. hookeri* var. *tricellularis*. Nine populations of *N. cristata* from New Zealand were identical to two populations from Victoria and another from New South Wales, Australia (Figure IV-4). Sister to these was a clade including several populations of *N. cristata* from New South Wales and *N. cristata* var. *diffusa*. The specimens in the New Zealand/Australia clade share specialized reduced primary branchlet segments that are packed with starch grains and appear opaque. These opaque segments (often called 'opaque dactyls') function as vegetative reproductive units, easily dehiscing and developing into new plants. The type specimen of *N. cristata* from Tasmania was examined by Michelle Casanova (personal communication) and discovered to differ from members in the New Zealand/Australia clade in vegetative morphology and oospore ornamentation. This distinction, along with the *rbcL* phylogeny, suggests that that *N. cristata* in New Zealand and some populations in southern Australia likely represent a new species. Further analysis of the *N. cristata* complex including chromosome counts, oospore ornamentation, and morphological descriptions, is currently underway and should help resolve the nomenclature for this taxon (Michelle Casanova, in prep.).

Excluding *N. cristata* and *N. hookeri*, eight species with only a few varieties and several forms are currently recognized for subgenus Hyella (Wood, 1965; Wood, 1972; Wood and Mason, 1977). Owing to the relative paucity of collections and little-understood morphological variation, this subgenus has not been further divided into more restrictive sectional groups (Wood, 1965; Wood, 1972; Wood and Mason, 1977). Of

these eight species, *rbcL* sequence data have been assembled here from thalli morphologically identifiable as *N. pulchella* Allen (Sakayama et al., 2004; Sakayama et al., 2002), *N. leptostachys* var. *leonhardii* (R.D.W.) R.D.W., *N. leptostachys* A. Br. em., *N. tasmanica* Müll. ex. A. Br. em., *N. tasmanica* ssp. *gelatinifera* R.D.W., and *N. tasmanica* ssp. *gelatinifera* var. *microcephala* (A. Br.) R.D.W. A further five unique *rbcL* sequences have been determined from plants that are either difficult to identify to species or correspond to no known species description (Figure IV-4; Appendix II).

The *rbcL* phylogeny shows a basal split separating the Japanese *N. pulchella* from all other members in this subgenus, which is exclusively comprised of Australia and New Zealand collections. Three distinct lineages were recovered in the Australia/New Zealand clade. (1) The *Nitella cristata*/*N. hookeri* clade discussed above. (2) A group including two identical isolates of *N. leonhardii* sister to three identical *N. leptostachys* isolates was recovered with an unidentified dioecious taxon (*Nitella* sp. 245). Both *N. leonhardii* samples were collected in New Zealand, while the three *N. leptostachys* were collected in New South Wales, South Australia, and Victoria, respectively. *Nitella* sp. 245 was collected in Tasmania and differs from *N. leonhardii* and *N. leptostachys* by being dioecious, though all three species share reduced fertile heads and dense mucus. (3) A weakly supported clade was recovered that includes two isolates of *N.* aff. *tasmanica*, *N. microcephala*, *N. gelatinifera*, and four unidentified *Nitella* species. Three of the unidentified *Nitella* species are monoecious (*Nitella* sp. 175, *Nitella* sp. 352, and *Nitella* sp. 353) and the fourth is dioecious (*Nitella* sp. 115). Limited structural material is available to resolve the taxonomy within this group, although several interesting trends are noted in the *rbcL* phylogeny. Most of the unidentified *Nitella* species fell within a

single clade. This clade was divided into two distinct biogeographic assemblages, one from eastern Australia, and another from southwest Australia. It is worth noting that southwest Western Australia is well known for its high level of vascular plant endemism (see *Flora of Australia* online http://www.deh.gov.au/biodiversity/abrs/online-resources/abif/flora/main/index.html). Many species are found only in that small area and an entire volume of *Eucalypts of Australia* is devoted to Western Australia species (REF), while the other volume covers the remainder of Australia. Given the occurrence of a separate clade of species from southwestern Western Australia, and the fact that the flora and diversity of that area are similar to that of the fynbos in South Africa, it will be interesting to compare *Nitella* collections from South Africa with the Western Australia collections. With more collections, the possibility exists that these unidentified isolates may be correlated with already published species. However, as it stands, it appears that they likely represent new taxa and warrant further research.

Another important point drawn out by the *rbcL* phylogeny for subgenus Hyella is the placement of *N.* aff. *tasmanica*. Three isolates corresponding to *N. tasmanica* were sequenced for *rbcL* and none proved to be closely related to another (Figure IV-4). Isolate F154 from Victoria and 355 from Western Australia were each found closely related to other taxa from similar biogeographic regions (see above). The third isolate collected in New South Wales was found sister to the *N. cristata*/*N. hookeri* assemblage, but the support for this placement was poor, likely due to the relatively long branch associated with it (Figure IV-4). The taxonomy of *N. tasmanica* is difficult to interpret. Mislabeled specimens, lost types, and poor illustrations have compounded this difficult problem (Wood, 1972). Tremendous morphological variation exists within *N. tasmanica*

ranging from minute simple forms, to diffusely branched forms with fertile heads, to robust heteromorphic forms with simple sterile branchlets and tiny fertile heads (Wood, 1972). The three isolates studied here are morphologically consistent with this general description, having diffusely branched sterile branchlets and reduced fertile heads. The *rbcL* data clearly place each *N.* aff. *tasmanica* isolate on separate evolutionary lineages and these data, along with the five unique unidentified isolates, further suggest that tremendous uncharacterized species diversity exists for subgenus Hyella.

Subgenus Tieffallenia

Based on dactyl cell number or end-cell shape, Wood (1965) divided subgenus Tieffallenia into eight sections, and included three species in an additional group of uncertain taxonomic affinity (Incertae). Phylogenetic studies based on *rbcL* (Sakayama et al., 2002) and *rbcL* and *atpB* combined (Sakayama et al., 2004) for subgenus Tieffallenia, however, found results that conflict with the sectional taxonomic system of Wood (1962; 1965). Using taxa biogeographically limited to Japan and Malaysia, Sakayama *et al*. (2004) showed that sections Tieffallenia and Gioallenia were polyphyletic, and further suggested that oospore membrane architecture was consistent within monophyletic groups of subgenus Tieffallenia. Results presented here using *rbcL* sequence data from a broad range of species further corroborate that sections Tieffallenia and Gioallenia are polyphyletic. These data also show that section Persoonia is paraphyletic and support a monophyletic Decandollea (Figure IV-5).

Section Decandollea, represented here by *Nitella hyalina* (DC.) Ag., *N. congesta* (R. Br.) A. Br., and *N. lhotzkyi* (A. Br.) A. Br. em., are often encrusted with calcium

carbonate and are commonly enveloped in thick mucus. They have short, 2-3 furcate branchlets with central secondary rays and strictly two-celled dactyls (Wood, 1965). Most notably, heteroclemous branchlets are found in all members of this section, though these species are not closely related to *N. stuartii* or *N. macounii* which also have a heteroclemous tendency (see '*n* = 6/12 (15) clade' discussion above). Members of section Decandollea also share similar fibrous oospore ornamentation (John and Moore, 1987; Nordstedt, 1891; Sakayama et al., 2004).

The *rbcL* results placed *Nitella ignescens* A. García, a recently described dioecious species from Australia (Garcia 1998), within section Decandollea, unresolved among an assemblage of *N. hyalina* and *N. lhotzkyi* isolates. *Nitella congesta* was strongly supported sister to these (Figure IV-5). *Nitella ignescens* shares with Decandollea mucus production, short 2-3 furcate branchlets, central secondary rays, and 2-celled dactyls. García (1998) collected *N. ignescens* from the uppermost littoral zone and reported plant size to be relatively small (3.0-5.0 cm) with mucronate dactyls, however, specimens provided by García from several localities in Australia grew over 20 cm tall in culture, reaching the top of the culture flask, and exhibited both mucronate and confluent dactyls similar to other members of Decandollea (not shown). Although a sectional affinity was not proposed, García (1998) noted that advantageous branchlets were observed inside one of the branchlet whorls, but because these branchlets were rare, she suggested that this was an abnormality rather than an expression of the heteroclemous condition. Advantageous branchlets were not observed in cultured material of *N. ignescens*, but heteroclemous branchlets are often lost in cultures of *N. hyalina* and especially *N. lhotzkyi* (personal observation) and may also have been lost in cultures of *N.*

ignescens. Further collections and culture-based experiments are needed to resolve the heteroclemous condition in *N. ignescens*. Unique to this section, *N. ignescens* possesses distinct reticulate oospore membrane ornamentation (García, 1998).

Dioecious *Nitella ungula*, described in the same publication as *N. ignescens* (García, 1998), was found sister to section Decandollea, although the *rbcL* sequence was quite divergent (Figure IV-5). *Nitella ungula* differs from members of section Decandollea in having distinctive, long end cells (to 270 μm) that sometimes are claw-like enclosing the gametangia. *Nitella ungula* lacks mucus, does not exhibit heteroclemous branchlets, and has vermiferous oospore ornamentation under SEM (García, 1998). Affinity of *N. ungula* with Decandollea can be found in the short 1-2 furcate branchlets, often with central secondary rays, and strictly bicellulate dactyls; however, the divergent *rbcL* sequence and unique dactyl morphology might warrant a new section for this species. The type was collected from Lake Bathurst, New South Wales, Australia, and also reported from Lake Muirhead, Victoria (García, 1998). The specimen used here for molecular analysis was collected by Michelle Casanova from Lake Bolac, Victoria, and is a new locality for this distinctive species.

Section Muelleria contains a single species, *Nitella partita* Nordst., and its phylogenetic placement has been problematic. *Nitella partita* is unique among *Nitella* species in that it has 2-3 partite dactyl end cells (Wood and Imahori, 1964). Based on illustrations alone, Wood (1965) assigned this species to its own section (Muelleria), and later expressed uncertainty when moving it to section Tieffallenia, near *N. furcata* (Wood, 1972). Results presented here strongly suggest that *N. partita* is not closely related to *N. furcata*, but rather is sister to a clade containing *N. verticillata* (section

Migularia), and an undescribed dioecious species (*Nitella* sp. 175) from Victoria, Australia.

Nitella verticillata (p254 KGK0113) was described by Filarszky (1937) as a new monotypic genus, *Charina*, using sterile and incomplete material provided by G. O. Allen, who was seeking an opinion of the material. James Groves thought it was a *Nitella* closely related to *N. subtilissima* (Filarszky, 1937), and Zaneveld (1940) thought that *Charina* was too badly defined to include in his treatment of the Characeae. Wood (1965; 1972) agreed that the available material was insufficient, but emphasized monopodal habit and acute dactyls to align this species in section Migularia, near *Nitella cristata*. *Nitella verticillata* was described as monoecious, though the type material was clearly sterile. Specimens are not abundant, and owing to its small size it is difficult to find. However, Wood (1972) inadvertently collected specimens with mature oogonia entangled with plants of *Chara fibrosa* Ag. ex Brux. em. from Western Australia and thereby suggested that *N. verticillata* may be dioecious. A recent discovery of *N. verticillata* from Brixton Street, N.R., Western Australia, Australia, collected by Michelle Casanova in 1996, clearly demonstrates that it is monoecious, with both male and female gametangia on the same plant, homeoclemous, with no calcification, mucus, or heads. Moreover, the *rbcL* sequence data do not show a close relationship of *N. verticillata* with *N. cristata* (discussed under *subgenus Hyella* above) but sister to dioecious *Nitella* sp. 175.

Nitella sp. 175 will be formally described by M. Casanova in a later paper. She kindly provided key morphological information for this species, which is discussed below. *Nitella partita*, *N. verticillata*, and *Nitella* sp. 175 share several morphological

features including central secondary branchlet segments and a small, delicate habit. *Nitella* sp. 175 differs from *N. verticillata* by its dioecious habit and end-cells that are not much reduced and are clearly not partite, but are confluent with the penultimate cell. The central secondary segment is reduced in *Nitella* sp. 175, giving a ragged monopodal appearance. All three species exhibit unique oospore ornamentation. *Nitella partita* has sparsely dispersed granulae and well developed flanges; *N. verticillata* has verrucate oospores and short flanges, and *Nitella* sp. 175 has densely packed granulate oospores without flanges.

It is impossible to reconcile the *rbcL* phylogeny with sections Tieffallenia, Gioallenia, and Persoonia *sensu* Wood (1965). Members of section Tieffallenia formed four distinct lineages dispersed among five lineages of Gioallenia and two lineages of Persoonia (Figure IV-5). Several clades, however, are consistent with those proposed by Sakayama *et al*. (2004) and correlate with oospore morphology. Sakayama *et al*. (2004) identified a clade including *N. pseudoflabellata* and *N. megaspora*, both with finely granulate oospores (termed FG clade). *Nitella leibergii* and two isolates of *N. confervaceae* from eastern United States were found nested among these taxa. Wood (1965) reported granulate oospores for *Nitella leibergii*, and SEM revealed *N. confervaceae* oospores to be granulate (data not shown). *Nitella confervaceae* has also been reported to be fibrous (John and Moore, 1987); however, the oospores examined in their study were from a French specimen labeled *N. batrachosperma*, an illegitimate name used for samples studied from that region (Hy, 1905; Hy, 1913; Hy, 1914). *Nitella mucosa* (Nordst.) J. Groves, *N. penicillata* Braun, *N. sonderi* Braun, and an unidentified *Nitella* species from China (*Nitella* sp. 251), formed a monophyletic group sister to the

FG clade. All species in this clade have granulate to papillate oospores (Leitch et al., 1990). It is interesting to note that both *N. penicillata* and *N. sonderi* are morphologically similar dioecious species distinguished by the production of mucus; *N. sonderi* produces mucus and *N. penicillata* does not. *Nitella* sp. 251, a monoecious taxon, was found embedded within three isolates of *N. sonderi* (Figure IV-5). *Nitella* sp. 251, like *N. sonderi*, produces mucus, but fertile branchlet segments are somewhat reduced after the primary branchlet ray, which maintains a length similar to sterile branchlets in culture (not shown). LM revealed a granulate oospore surface like other species in this clade, but oospores from this species should be reexamined under SEM to confirm the surface ornamentation.

A second clade identified by Sakayama *et al.* (2004) included species with reticulate or papillate oospore membrane (RP clade; *N. furcata* (Roxb. ex Bruz.) Ag., *N. inversa* Imah., *N. tumulosa* Zanev., *N. gracillima* T.F. Allen, *N. axillaris* A. Br, and *N. axilliformis* Imah.). A clade including these species was also recovered in the analyses presented here, but this clade now includes *N. megacarpa* T. F. Allen, *N. polycarpa* Pal., *N. intermedia* Nordst. in T. F. Allen, and *N.* aff. *mucronata* (Figure IV-5). The monophyly of this group was poorly supported with the *rbcL* data, and additional data from *atpB* was required to obtain reasonable support values for the RP clade (Sakayama et al., 2004). *Nitella megacarpa*, *N. polycarpa*, *N. intermedia*, were consistent with all sharing papillate or irregular reticulate oospore surface under LM. SEM studies of *N. megacarpa* and *N. intermedia* also showed papillate or reticulate ornamentation (Cáceres, 1977; John and Moore, 1987). Because voucher material was not available for *N.* aff.

mucronata, oospores were not examined and the species determination of this isolate is in doubt.

A third clade described by Sakayama *et al*. (2004) was characterized by finely granulate oospore membrane and strongly flanged ridges (VFG clade). This clade included several isolates of *Nitella gracilens* Morioka (Sakayama et al., 2004). In this study, *N. subtilissima* A. Br. em was found sister to a clade containing *N. gracilens* and *N. orientalis* T. F. Allen (Figure IV-5). *Nitella gracilens*, *N. orientalis* and *N. subtilissima* share prominent flanged ridges and the oospore membrane of *N. subtilissima* is granulate (Frame, 1977), while *N. orientalis* has obscurely granulate (Wood, 1965) or roughened, pitted, or papillate oospore ornamentation (Frame, 1977). Sister to the VFG clade was an assemblage of three species with variable oospore morphology, *N. partita*, *N. verticillata*, and *Nitella* sp. 239 (discussed above).

Six populations of *N. tenuissima* (Desv.) Kuetz. were sampled. One from Indiana, two from Michigan, and three from Wisconsin, and *rbcL* sequence data for all populations were found to be identical. This species formed a distinct lineage near the base of subgenus Tieffallenia, not closely allied with any other species sampled (Figure IV-5). Consistent with its distinct phylogenetic placement, oospores of *N. tenuissima* are strongly beaded reticulate (Frame, 1977; John and Moore, 1987), an uncommon condition among species in this subgenus.

The final clade identified by Sakayama *et al*. (2004) included *N. spiciformis* Morioka and *N. moriokae* R.D.W. both with tuberculate oospore membrane (TUB clade). A clade containing these two species along with *N. translucens* (Pers.) Ag. and *N. mucronata* (A. Br.) Miq. was recovered with strong support (Figure IV-5). Similar to *N.*

spiciformis and *N. moriokae*, *N. translucens* and *N. mucronata* have strongly flanged oospores, however the oospore surface is described as finely reticulate due to low anastomosing ridges or surface wrinkles in *N. translucens*, and strongly reticulate for *N. mucronata* (John and Moore, 1987).

Conclusions

Determination of the taxonomy and molecular phylogeny within the genus *Nitella* is not altogether a simple problem. However, results like those presented by Sakayama and his colleagues (Sakayama et al., 2004; Sakayama et al., 2002), and those presented here, serve as steps toward untangling the complex evolutionary history of *Nitella*. Prior to the application of contemporary molecular method, charophytologists have painstakingly tried to reconcile a diverse and morphologically variable group of fresh water green algae into a clear and natural system. The molecular results presented here, combined with previously published data, have resolved a few questions within *Nitella* and raised even more. A basal split in *Nitella* revealed two clades that correlate with chromosome number, while single-celled dactyls likely represent a pliesiomorphic condition not useful in defining deep divergences in this genus. All taxa with multicelled dactyls (≥ 2) studied to date form a strongly supported, natural group. This group can further be divided into two lineages, one characterized by 2(-3) celled dactyls (Tieffallenia) and the other by ≥ 3 celled dactyls (Hyella). Most previous authors agreed with this latter finding, and the proposed exceptions have proven to not be exceptions after all (e.g., *Nitella cristata* and *N. tasmanica*). Thorough collection of members of subgenus Hyella promises to reveal considerable unrecorded species diversity. SEM

studies of oospore morphology are remarkably well correlated with the molecular phylogeny providing synapomorphies for relatively finite groups of *Nitella* species.

Are multiple representatives of morphologically similar *Nitella* accessions different species? These current data do not allow us to strongly invoke any one existing species concept to resolve this question. Application of the biological species concept (Dobzhansky, 1935; Mayr, 1942) would require reproductive compatibility data that are not available for *Nitella*, though several studies exist for *Chara* (see Chapter I). *Nitella* are generally delicate and difficult to culture. Crossing experiments would require large amounts of green house space and sufficient time for completion of their lifecycle. Though feasible, testing the biological species concept for *Nitella* can only be seen as project with a long-term goal of generating adequate reproductive compatibility data. Application of the phylogenetic species concept (Cracraft, 1989; de Queiroz and Donoghue, 1990) would require additional molecular markers from multiple accessions that represent populations of *Nitella* species. Collection of multiple *Nitella* species is currently under way and sequencing of a complete nuclear genome of *Chara braunii* is in progress (as part of the NSF Tree of Life program). With a completed genome and multiple isolates of *Nitella* available, testing the phylogenetic species concept appears tractable. This leaves the morphological species concept (Mayr, 1942). Morphological variation within *Nitella* has resulted in a troublesome taxonomic history. Increased taxon sampling guided by robust phylogenies offers hope toward understanding this variation and identifiying key morphological characters useful for understanding this interesting group of green algae.

Appendix I. Clade names and definitions. Many of the relationships (clades) presented for the early branching events in the Charophyta were unresolved prior to this study. As a consequence formal names for these clades were unavailable. Clades were assigned names loosely following the PhyloCode emphasizing node-based rather than stem-based names. Node-based definition was preferred because much of the discussion regarding these clades revolves around the ancestor more so than the stem lineage. Contrary to the PhyloCode, several clades were identified by generic rather than species names either because species were difficult to identify or DNA sequences were derived from different species of the same genus. These names are not meant to be formal assignments, rather a temporary means of communication in the context of this dissertation. I strongly feel that formal names are needed, but further study is necessary.

[Volvox...Chlam] is the least inclusive clade containing *Volvox carteri* and *Chlamydomonas reinhardtii*.

[Volvox...Paulsc] is the least inclusive clade containing *Volvox carteri* and *Paulschulzia pseudovolvox*.

[Volvox...Pterom] is the least inclusive clade containing *Volvox carteri* and *Pteromonas angulosa*.

[Klenit...Klesub] is the least inclusive clade containing *Klebsormidium nitens* and *Klebsormidium subtilissimum*.

[Klenit...Klefla] is the least inclusive clade containing *Klebsormidium nitens* and *Klebsormidium flaccidum*.

[Klebsormidiales] is the least inclusive clade containing *Klebsormidium nitens* and *Entransia fimbriata*.

[Mesota...Mougeo] is the least inclusive clade containing *Mesotaenium caldariorum* and *Mougeotia sp*.

[Spirog...Zygnem] is the least inclusive clade containing *Spirogyra maxima* and *Zygnema peliosporum.*

[Mesota...Zygnem] is the least inclusive clade containing *Mesotaenium caldariorum* and *Zygnema peliosporum.*

[Cosmoc...Onycho] is the least inclusive clade containing *Cosmocladium perissum* and *Onychonema sp*.

[Cosmoc...Gonato] is the least inclusive clade containing *Cosmocladium perissum* and *Gonatozygon monotaenium*.

[Cirreg...Csiemi] is the least inclusive clade containing *Coleochaete irregularis* and *Coleochaete sieminskiana*.

[Corbic...Csolut] is the least inclusive clade containing *Coleochaete orbicularis* and *Coleochaete soluta*.

[Lychno...Nitops] is the least inclusive clade containing *Lychnothamnus barbatus* and *Nitellopsis obtusa*.

[Lampro...Cconni] is the least inclusive clade containing *Lamprothamnium macropogon* and *Chara connivens*.

[Lampro...Caustr] is the least inclusive clade containing *Lamprothamnium macropogon* and *Chara australis*.

[Trache...Sphagu] is the least inclusive clade containing tracheophytes and *Sphagnum spp*.

[Trache...Anthos] is the least inclusive clade containing tracheophytes and *Anthoceros*.

[Embryo...Charales] is the least inclusive clade containing embryophytes and Charales.

[Embryo...Coleochaetales] is the least inclusive clade containing embryophytes and Coleochaetales.

[Embryo...Zygnematales] is the least inclusive clade containing embryophytes and Zygnematales.

[Embryo...Klebsormidiales] is the least inclusive clade containing embryophytes and Klebsormidiales

[Embryo...Chlorokybales] is the least inclusive clade containing embryophytes and *Chlorokybus*.

Appendix II. Summary of species and source information for Characeae. Vouchers for collections with a numbers preceeded by 'KGK' have been deposited in the University of Maryland Norton-Brown Herbarium (MARY). GenBank numbers for previously published data and locality information for new data is provided when known.

Taxon	collection or GenBank number	other strain or collection number	Locality
Chara			
C. australis		X-067	Outside Sydney, New South Wales, Australia
C. braunii	KGK-0339	589	Mrs. L's Playa, Lubbock, Texas, USA
C. brittonii	KGK-0196	X-997	Lawrence Lake, Barry, Michigan, USA
C. connivens	AF097161		
C. fibrosa	KGK-0057a		Taihu Lake, Wuxi, Jiangsu, China
C. haitensis		TAMPS 80	Xicotemcatl, Tamalpais, Mexico
C. vulgaris	AF097166	X-152	Demark
Lamprothamnium			
L. heraldi	KGK-0069	AG-22-10-01-8	Lower Bell Lake, New South Wales, Australia
L. macropogon	U27534		Tasmania, Australia
L. papulosum	AF097170		France
Lychnothamnus			
Ly. barbatus	AF097172		Poland
Ly. barbatus	U27533		pond near Bacinska, Jezera, Croatia
Ly. barbatus	AF097171		Wallace Creek, Queensland, Australia
Nitellopsis			
Ni. obtusa	U27530		Stadtwaldsee, Bremen, Germany
Ni. obtusa	KGK-0057e		Taihu Lake, Wuxi, Jiangsu, China
Tolypella			
T. glomerata	AF097176		
T. nidifica	U27531		
T. porteri		X-907	
T. prolifera	AF097175		
T. prolifera		F142	
T. stipitata		Sue Miers	
Nitella			
N. acuminata	KGK-0052a		Long Lake, Iron, Wisconsin, USA
N. acuminata	KGK-0346	159	Arenal Road, Costa Rica
N. acuminata	AB110866		
N. axillaris	KGK-0216	152B	Lake Apanas, Nicaragua
N. axilliformis	AB110877		Watarase-yusuiti, Tochigi, Japan
N. bastinii		773	Floyd, Texas, USA
N. clavata	KGK-0232	X-770 = Peru 3B	Chincheras #2, near Cuzco, Peru
N. confervacea	KGK-0037c		Lake Burnt Mills, Isle of Wight, Virginia, USA

N. congesta	KGK-0354	p462	Bannister River, Western Australia, Australia
N. cristata	KGK-0277	12/NC/-/Brunner	Lake Brunner, South Island, New Zealand
N. cristata	KGK-0128	p266	Barleyfields Lagoon, New South Wales, Australia
N. cristata	KGK-0118	p259	Dumaresq Creek, New South Wales, Australia
N. diffusa	KGK-0368	p471	Ararat, Victoria, Australia
N. flexilis	KGK-0392		Partin's Mill Pond, Wake, North Carolina, USA
N. furcata	AB076059		
N. furcata	KGK-0038		Airfield Lake, Sussex, Virginia, USA
N. gelatinifera	KGK-0270	AG-17-2-02-1	Cunning River, Western Australia, Australia
N. gracilens	AB110870		Yokawa-cho, Hyogo, Japan
N. gracilens	AB076061		Japan
N. gracillima	AB110874		Yokawa-cho, Hyogo, Japan
N. hookeri	KGK-0121	2/NH/-/Raira	Lake Rotoaira, Central North Island, New Zealand
N. hookeri	KGK-0121	2/NH/-/Raira	Lake Rotoaira, Central North Island, New Zealand
N. hyalina	KGK-0190	706	Lake Skadarsko, Montenegro, Yugoslavia
N. hyalina	AB076067		Japan
N. hyalina	KGK-0227	AG-22-10-01-11	Wallace Creek, Queensland, Australia
N. hyalina	KGK-0328	AG-22-11-01-1	Lake Fitzpatrick, Australia
N. hyalina	KGK-0271	Nhy/1/-/Tarawera	Lake Tarawera, North Island, New Zealand
N. hyalina	KGK-0059b	Y-030	Lake Mattamuskeet, Hyde, North Carolina, USA
N. ignescens	KGK-0258	AG-30-09-01-3	Greens Lake, Victoria, Australia
N. ignescens	KGK-0258	AG-30-09-01-1	Lake Cooper, Victoria, Australia
N. inversa	AB076060		Japan
N. leibergii	KGK-0051		Sandy Beach Lake, Iron, Wisconsin, USA
N. leonhardii	KGK-0119		Lake Rotoma, North Island, New Zealand
N. leptostachys	KGK-0240	p299	Freshwater Lake, Grampians, Victoria, Australia
N. lhotzkyi	KGK-0287	p404	Caroline Springs, Melbourne, Victoria, Australia
N. macounii	KGK-0156	235A	Belle Plaine, Saskatchuwon, Canada
N. megacarpa	KGK-0052b		Long Lake, Iron, Wisconsin, USA
N. megaspora	AB110872		Watarase-yusuiti, Tochigi, Japan
N. microcephala	KGK-0351	p467	Piney Lake, Western Australia, Australia
N. mirabilis	AB110865		Bog at Yokawa-cho, Hyogo, Japan
N. misouriensis		X-867	New Mexico, USA
N. moriokae	AB110876		Yokawa-cho, Hyogo, Japan
N. mucosa	KGK-0275	NP/1/-/Okataina	Lake Okataina, North Island, New Zealand
N. mucronata		F109	France
N. mucronata	KGK-0189	705	Lake Skadarsko, Montenegro, Yugoslavia
N. aff. Mucronata		P/CR1	Panama/Costa Rica
N. opaca	AF097174	F146	
N. opaca	KGK-0276	NF/1/-/Tarawera	Lake Tarawera, North Island, New Zealand
N. orientalis	KGK-0291	p378	Murray River, South Australia, Australia
N. partita	KGK-0176	p279	Pied Stilt Swamp, New South Wales, Australia
N. penicillata	KGK-0174	p281	Olympic wetland, Victoria, Australia
N. polycarpa	KGK-0230	150B	Lake Apanas, pearched pond, , Nicaragua
N. praelonga	KGK-0390		Lake Okeechobee, Florida, USA
N. pseudoflabellata	AB076065		Japan
N. pseudoflabellata	AB076066		Japan
N. pulchella	AB076057		Japan
N. sonderi	KGK-0292	p379	Murray River, South Australia, Australia

N. sonderi	KGK-0251	AG-13-01-02-2	Paddy's River, New South Wales, Australia
N. sp.	KGK-0245	p296	Interlaken, Tasmania, Australia
N. sp.	KGK-0175	p280	Halls Gap, Grampians, Victoria, Australia
N. sp.	KGK-0115	p256	Native Dog Creek, New South Wales, Australia
N. sp.	KGK-0352	p465	Margaret River, Western Australia, Australia
N. sp.	KGK-0353	P464	Bootjidup River, Western Australia, Australia
N. sp.	KGK-0239	p298	Freshwater Lake, Grampians, Victoria, Australia
N. sp.	KGK-0295		Lake Habeeb , Allegany, Maryland, USA
N. sp.	KGK-0057g		Taihu Lake, Wuxi, Jiangsu, China
N. spiciformis	AB110875		Watarase-yusuiti, Japan
N. stuartii	KGK-0273	NS/1/-/Acheson	Acheson Stream, North Island, New Zealand
N. stuartii	KGK-0382	AG-30-12-02-2-3	Molonglo River, New South Wales, Australia
N. stuartii	F155b	F155	Dumaresq Creek, New South wales, Australia
N. subtilissima	KGK-0082	p231	Lanark, Hamilton, Victoria, Australia
N. aff. tasmanica	F156	F154	Barleyfields Lagoon, New South Wales, Australia
N. aff. tasmanica	KGK-0355	p466	Mt. Barker, Western Australia, Australia
N. aff. tasmanica	KGK-0078	AG04	Paddy's River, New South Wales, Australia
N. tenuissima	KGK-0049b		Lake Tomohawk, Oneida, Wisconsin, USA
N. translucens	AF097745	F108	France
N. tricellularis	KGK-0186	14/NH/T/Lyndon	Lake Lyndon, South Island, New Zealand
N. tumulosa	AB110868		Haew Loam waterfall, Thailand
N. ungula	KGK-0173	p282	Lake Bolac, Victoria, Australia
N. verticillata	KGK-0113	p254	Brixton St NR, Western Australia, Australia

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