ABSTRACT

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DIVERSITY OF CONJUGATING GREEN ALGAE; PHYLOGENETIC STUDIES OF A SPECIES-RICH EUKARYOTIC LINEAGE

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This thesis used molecular phylogenetic techniques to investigate diversity in the conjugating green algae. The conjugating green algae are closely related to land plants and evolution within the group provides a good analogy of how evolution may have proceeded in the lineage that gave rise to land plants. I developed a dataset of the genes *cox*III, *psaA* and *rbcL* with 109 taxa to determine phylogenetic relationships of the families and genera. I found that the order Zygnematales is not monophyletic and that *Spirogyra* was the first to branch. The order Desmidiales is monophyletic if one includes the genus *Roya*. The family Peniaceae is not monophyletic. The genera *Cosmarium*, *Cylindrocystis*, *Mesotaenium*, *Penium*, *Spondylosium*, *Staurodesmus* and in later studies *Desmidium* and *Hyalotheca* were found to be paraphyletic or polyphyletic.

Investigation of cell division syndromes among filamentous Desmidiaceae revealed greater diversity than was previously reported. Notable among these discoveries is that *Spondylosium pulchrum* displays the *Desmidium*-type cell division, *Spondylosium pulchellum* the *Cosmarium*-type, and *Spondylosium tetragonum* the newly described *Teilingia*-type cell division. The relationship among the syndromes was inferred from

phylogenetic analysis of the species that revealed a single lineage comprising filamentous and colonial species and multiple modes of cell division. This suggests that even the fundamental process of cell division can be highly modified. Results from this study also resulted in the taxonomic resurrection of the genus *Didymoprium*, as well as the creation of the new genus *Isthmocatena* and the combinations *Didymoprium grevillei*, *Desmidium pulchrum*, and *Isthmocatena pulchella*.

Investigations of the Gonatozygaceae revealed unexpected diversity in *Gonatozygon brebissonii* and *G. kinahani*. Structural measurements were sufficient to distinguish among strains of *Gonatozygon* species except for *Gonatozygon brebissonii*. We have probably underestimated genetic and species diversity in this family. In contrast, the structurally distinct species *Triploceras gracile*, was found to be closely related to *Micrasterias*. This relationship was confirmed by sequencing and phylogenetic analysis of the nuclear encoded EF1α, EIF4 and TUA. The results of this study indicate that *Triploceras* is probably actually bilaterally symmetric, although it has been treated as a radially symmetric species.

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Chapter 1. General Introduction

Early land plant evolution

The colonization of land by plants was one of the most important events in the history of modern life on Earth. Before the advent of land plants, photosynthesis was likely restricted to aquatic and damp terrestrial habitats. Land plants, like their aquatic counterparts, consume carbon dioxide, fix and sequester the carbon in complex organic molecules, and release oxygen into the atmosphere. Today, terrestrial plants account for 56.4% of total net primary production (Field et al., 1998). Plants not only contributed to the total production of photosynthate on Earth, but were also responsible for building soils and their success generated new habitats for other organisms.

It is easy to speculate on the evolutionary developments that gave rise to the modern land flora once these organisms were established on land, but the nature of the photosynthetic pioneers remains obscure. What characteristics – cellular, sexual, cytoplasmic and genetic – may have favored their success? In animals, evolution of the modern fauna is comparatively well documented in the fossil record. Early land plants, apparently, had few degradation resistant parts and are not well represented in the fossil record.

Some of the oldest fossil plants suggest that the origin of the land flora is at the latest 475 mya (Wellman et al., 2003). These fossils resemble extant mosses. Of course, the colonization of land may have occurred long before these plants were fossilized. It is now widely accepted that land plants evolved from one lineage of green algae, commonly known as the charophytes (Mattox and Stewart, 1984; Huss and Kranz, 1995; Kranz et al., 1995; Lewis and McCourt, 2004; McCourt et al., 2004).

Charophyte green algae

There are at least six major lineages of extant charophyte green algae (Lewis and McCourt, 2004), which will be here discussed as classes of the division Charophyta. These include the Mesostigmatophyceae, Chlorokybophyceae, Klebsormidiophyceae, Zygnematophyceae (conjugating green algae), Coleochaetophyceae and the Charophyceae *sensu stricto* (stoneworts). Systematic relationships, fossil record and general characteristics of these lineages are discussed in Chapter 2. Charophytes are united by a number of cytological characteristics. Most of the structural evidence for the relatedness of charophyte algae and land plants is present in the motile cells. *Chara* and *Coleochaete* produce motile sperm while *Coleochaete*, *Klebsormidium* and *Chlorokybus* produce flagellate zoospores. *Mesostigma viride* is itself a biflagellate cell. These flagellate cells all have a subapical insertion of two isomorphic flagella (Pickett-Heaps and Marchant, 1972; Mattox and Stewart, 1984; Melkonian, 1989). These flagella are rooted with a characteristic associated multilayer structure (MLS) at the base. The MLS is a dense array of lamellar structures that attach to the basal bodies of the flagellar apparatus. While most charophytes have a single MLS, *Mesostigma viride* has two MLSs (Rogers et al., 1981; Melkonian, 1989). Although the MLS in green algae is unique to the charophytes, a structure similar to a MLS has been found in other unrelated organisms.

The conjugating green algae are conspicuously without a flagellate stage in the life cycle and their inclusion in the charophyte green algae was based on the presence of a persistent mitotic spindle (Pickett-Heaps and Marchant, 1972; Mattox and Stewart, 1984). Since the recognition of charophytes as a lineage unto themselves, other characteristics have been found which seem to unite the group or some members of the group with land plants. Among the charophytes investigated, all seem to have cellulose synthesizing rosettes, circles of cellulose synthetase protein complexes, as opposed to linear arrays of synthetase protein complexes found in most chlorophyte green algae (Domozych et al., 1980; Hotchkiss and Brown, 1987; Okuda and Brown, 1992; Tsekos, 1999). Different arrangement of the synthetase complexes translates to different numbers of cellulose microfibers and differently shaped cellulose fibers in the cell walls. Arguably, slight differences in cell wall composition could correlate to differences in cell-to-cell communication, structural strength, or other factors that may have given charophyte green algae an advantage in colonizing terrestrial habitat.

In addition to cellulose synthesizing rosettes, some charophytes also have an unique variant of cell division that involves a microtubular array called a phragmoplast (Mattox and Stewart, 1984; Cook et al., 1998; Cook, 2004b). The phragmoplast forms at the time of cytokinesis and seems to be involved with the deposition of primary wall in a cell plate. In the case of *Chara*, *Coleochaete* and land plants, the cell plate is deposited from the center of the cell toward the periphery of the cell (Cook et al., 1998; Cook, 2004b). All other charophytes divide by the centripetal encroachment of a division septum. In this case, the cell wall forms from the periphery of the cell toward the center without the association of a highly organized microtubular array (Bech-Hansen and Fowke, 1972; Floyd et al., 1972; Pickett-Heaps, 1972; Rogers et al., 1980; Mattox and Stewart, 1984). This would seem to set *Chara* and *Coleochaete* apart as close relatives of land plants, however, some conjugating green algae also use a phragmoplast-like microfilament array (Fowke and Pickett-Heaps, 1969a; Fowke and Pickett-Heaps, 1969b; Pickett-Heaps and Marchant, 1972). This has only been shown in *Spirogyra*, and was later demonstrated to be functionally and compositionally different from a phragmoplast (Sawitzky and Grolig, 1995). Still, its presence in one of the earliest diverging lineages of conjugating green algae suggests that the phragmoplast may have been lost in the conjugating green algae. Alternatively, the actin-based microfilaments may have preceded the microtubule array used in plant phargmoplasts.

Besides structural evidence, a number of phylogenetic studies suggest that the charophytes are closely related to land plants. Three lineages, individually or in combination, have been proposed as the closest relative of land plants: Charophyceae, Coleochaetophyceae and Zygnematophyceae (Manhart and Palmer, 1990; Huss and Kranz, 1995; Kranz et al., 1995; Bhattacharya and Medlin, 1998; Nickrent et al., 2000; Karol et al., 2001; Turmel et al., 2003, 2006). Any of these hypotheses would be consistent with the structural evidence available and, at the present, there is little consensus among the competing hypotheses, however only these three lineages seem to be likely candidates based on presently available data.

Of these three lineages, one exhibits a degree of taxonomic and structural diversity comparable to that of land plants: the conjugating green algae (Zygnematophyceae). The extant Charophyceae are the remnants of a once more diverse lineage of macrophytes (Feist and Grambast-Fessard, 1991). The thallus of extant Charophyceae consists of a series of branched nodes and internodes. The internodes are single, large, multi-nucleate cells. The nodes, however, are made of many smaller cells with some being specialized cells. The branches attach in whorls at the nodes. Charophyceans have rhizome-like shoots that penetrate sediments as well as rhizoids and specialized reproductive structures, oogonia and antheridia. Extant Characeae are all oogamous, but may be hetero- or homothallic (Wood and Imahori, 1965).

The Coleochaetophyceae are branched epiphytic filaments. These organisms have a specialized hair cell that extends above the cell into the surrounding medium. Coleochaetophytes produce both zoospores and flagellate sperm (Delwiche et al., 2002). Sexual reproduction is oogamous, and the oogonia may be completely or only partially surrounded by jacket cells called cortical cells (Szymańska, 1988; Delwiche et al., 2002; Szymańska, 2003). There are fewer than thirty known species of Coleochaetophyceae and only a couple hundred species of Charophyceae (Chapter 2). These numbers are dwarfed by the estimated 350,000 land plant species that have been described in the literature.

Biology and systematics of the Zygnematophyceae

The conjugating green algae, unlike other charophyte lineages, include several thousand extant species (Hoshaw et al., 1990; Gerrath, 1993). These organisms are not just taxonomically diverse, but they are also phyletically diverse. A phylogenetic analysis of just the gene *rbcL* from sequences of charophyte green algae and land plants reveals that the branch lengths within the conjugating green algae are both far greater than those within the Charophyceae and Coleochaetophyceae, but also that the family Desmidiaceae shows almost as much sequence divergence as angiosperms (Figure 5.1). The fundamental biological differences among conjugating green algae defy generalizations.

The group includes organisms that are unicellular, colonial, and filamentous. Conjugating green algae can be found in nearly every freshwater habitat on every continent. They can be found on glaciers, in thermal pools, in acidic rivers and alkaline streams (Brook, 1981). Members of the group are often considered biological indicators of environmental conditions. For example, *Mougeotia* is sometimes an indicator of acidification, and *Spirogyra* an indicator of eutrophication. One group of zygnematophytes, the desmids, has been used more extensively in estimation of the geochemical trophic state and general water quality of freshwater habitats (Jarnefelt, 1952; Rawson, 1956; Brook, 1965; Vinebrooke and Graham, 1997; Coesel, 2001).

Zygnematophytes demonstrate a number of different sexual reproductive syndromes including oogamy, heterogamy and isogamy. They may be homothallic or heterothallic (Brandham and Godward, 1965). The presence of such fundamental differences among conjugating green algae makes them an excellent model system for understanding the basis of these syndromes in structurally simple organisms.

A number of cytological differences exist among the conjugating green algae. The most striking aspect of these organisms is their spectacular chloroplasts. These organelles are often very large and have complex shapes. The spiraling chloroplasts of *Spirogyra*, the flat chloroplasts of *Mougeotia*, which twist and turn in response to light stimulus, and the stellate chloroplasts of *Zygnema* are among the most recognizable chloroplasts of all green algae. *Spirogyra* is not the only familiar conjugating green alga, however. The desmids (order Desmidiales) are also frequently studied in introductory biology courses. The desmids are best known for their exceptionally intricate and decorated cell walls, however their chloroplasts are equally ornate.

The appearance of the cell walls by light microscopy does not demonstrate the complexity of these structures. Even apparently similar organisms may have cell walls that are structurally very different from one another. These differences are the basis of the order and family-level classification. One order, the Zygnematales, is characterized by a homogenous cell wall that is structurally similar to that of land plants, *Coleochaete* and *Chara* (Mix, 1972; Domozych et al., 1980; Hotchkiss et al., 1989). The cell wall consists of an extracellular primary cellulosic cell wall and a secondary wall that is deposited inside the primary wall. In some Zygnematales, there is also an extramural matrix, consisting partially of pectins and glycoproteins (Mix, 1972; Domozych et al., 1980; Hotchkiss et al., 1989).

In the order Desmidiales, the differences in cell wall characteristics define the families (Mix, 1972). In the Closteriaceae, Gonatozygaceae and Peniaceae, all of the ornamentation of the mature cell wall is actually present in the extramural matrix. Briefly, the extramural matrix consists of variously shaped fibrils that are arranged in a mesh-like network (Peniaceae and Gonatozygaceae) or more orderly lines (Closteriaceae) (Mix, 1966, 1969, 1972). Kouwets and Coesel (1984), found differences between the cell walls of *Penium* and *Gonatozygon* to be so trivial that they proposed merging those taxa into a single family, the Peniaceae. The family Desmidiaceae, which contains the majority of desmid species, has a very different cell wall structure.

In the Desmidiaceae, there are several different syndromes of cell division (investigated in Chapter 3) but there are general characteristics of the cell wall that are present in most taxa. In the case of Desmidiaceae, the primary wall has comparatively little ornamentation. During development of the cell wall, the secondary wall, with the characteristic ornamentation, is deposited inside the primary cell wall. Once mature, the primary wall is completely or partially shed leaving only the secondary wall along most of the cell. Sometimes an extramural matrix is deposited outside the secondary wall. These cell walls also have a more complex kind of pore than is found in the other families of desmids. In the Closteriaceae, Gonatozygaceae and Peniaceae, the pores are actually pits in the extramural matrix, which sometimes extend into the primary wall, but never penetrate the secondary wall. In the Desmidaceae, however, the pores completely traverse the secondary wall and sometimes have a complex associated protein apparatus (Mix, 1972).

Systematic treatment of the genera is heterogenous across the conjugating green algae. Among the Zygnematales, concepts of the genera are based on growth form (unicellular versus filamentous) and the shape of the chloroplast (Czurda, 1932; Transeau, 1951; Prescott et al., 1972; Kadlubowska, 1984). Among the Desmidiales, most generic concepts are based on differences in cell shape: presence or absence of processes, general shape of the cell, presence of apical lobes or incisions, presence, nature and distribution of ornamentation, etc. The importance of most of these characteristics has been directly contested in the literature resulting in the merging or splitting of groups of species into larger or smaller genera (Prescott et al., 1975; Compère, 1976; Palamar-Mordvintseva, 1976; Prescott et al., 1977; Prescott et al., 1981; Prescott et al., 1982; Croasdale et al., 1983; Compere, 1996; Palamar-Mordvintseva, 2003, 2005). At the moment, the general trend seems to be in favor of separating species with fundamentally different structural characteristics into different genera. For example, the genus *Haplotaenium* was recently separated from *Pleurotaenium* when it was found that one group of species lacked a terminal vacuole, has axile as opposed to parietal chloroplasts as well as a different distribution of pores on the cell wall (Bando, 1988). Similarly, the genus *Tortitaenia* was split from *Spirotaenia* based on the difference in chloroplast distribution (axile versus parietal) (Brook, 1998). These are just two of many examples.

Characters used to identify species vary among the zygnematophyte lineages. Among the Zygnematales, species delimitation hinges on differences in chloroplast distribution in the cell as well as the characteristics of the zygospore walls (Czurda, 1932; Transeau, 1951; Kadlubowska, 1984; Rundina, 1998). This makes identification of field material difficult because sexual cells are infrequently encountered during most of the year. Among the Desmidiales, species are based on differences in cell shape and ornamentation, however both are known to be plastic within species. Taxonomy of Desmidiales is complicated by a long history of the use of subspecies, varieties and forms. For many desmid species, this means that there may be dozens of subspecific names associated with the specific epithet (West and West, 1904; Prescott et al., 1972; Prescott et al., 1975, 1977; Prescott et al., 1981; Prescott et al., 1982; Croasdale et al., 1983). The basic concept of a species also differs among genera and investigators. In many cases, the biological species concept is inappropriate because the species is asexual, or not testable because it is not known how to grow the organism, induce sexual reproduction or induce germination of the resulting spores. In some groups, species are thought to exist in breeding complexes (ie. species complex) (Denboh et al., 2003a). In others, it is thought that many of the organisms described as varieties are very likely biological species.

Understanding diversity of the conjugating green algae

Given this bewildering diversity and inconsistent taxonomic treatment, how is one to interpret previous cytological and developmental studies or make any inferences about evolution within the conjugating green algae, much less about the lineage that gave rise to land plants? Investigation of these characteristics in a systematic and phylogenetic context would make it possible to both order the known information and would indicate where information about the organisms is lacking. This was the underlying motivation of this thesis.

At the initiation of this dissertation work, very little was known about the phylogenetic relationships of conjugating green algae. Studies at that time had been based on very few taxa and only a single gene, *rbcL* (Park et al., 1996; McCourt et al., 2000). These phylogenies were not well supported statistically and lacked many of the genera and species for which structural information was known. To address this, I first completed a phylogenetic study of the conjugating green algae using multiple genes and then investigated some of the gaps in our knowledge of the basic biology of these organisms with an aim to better understand their diversity and evolution.

Diversity has many definitions. When discussing "diversity," most scientists are referring specifically to species or taxonomic diversity, sometimes called biodiversity. Often, scientists are focused on the number and identity of species present in a particular location. There are other kinds of diversity, however, which may be pertinent to questions of evolution. Some other possible measures of diversity include phyletic, ontogenetic, structural, ecological and physiological diversity. In the case of conjugating green algae, structural diversity is closely related to taxonomic diversity because differentiation of species relies almost exclusively on structural characteristics.

Structural and taxonomic diversity are not necessarily directly related to other measures of diversity. Based on structural characteristics, some genera of conjugating green algae contain over 1,000 species, but our interpretation of their evolution would be very different if they were found by molecular phylogenetic investigation to all be part of a very recent radiation with very short shallow branches as opposed to a very deeply branching clade, or if species in this genus were polyphyletic with respect to other genera. In this hypothetical genus with a thousand species, the phyletic diversity may be congruous with the taxonomy of the group but a phylogenetic analysis can reveal differences among lineages that are relevant to our understanding of evolution of the group. Similarly organisms that have similar gross structural characteristics may have different physiological responses to environmental stress, different developmental pathways or different environmental niches.

Each measure provides different information about the group being studied. Much energy has been spent on structural diversity of the cell walls of conjugating green algae and significantly less emphasis has been placed on the characteristics of the cytoplasm including the chloroplast. Additionally, much information on the ecological diversity of the conjugating green algae exists, although the interactions between species and their environment on a physiological and biochemical level remains obscure. In this work, I investigated not only taxonomic diversity, but phyletic diversity, ontogenetic diversity and structural diversity in a molecular systematic context. By doing so, I hoped to increase our understanding of the diversity of the conjugating green algae and provide some insight into how different characteristics evolved in the conjugating green algae and, possibly in the lineage that gave rise to land plants.

This thesis contains five chapters based on newly collected data (Chapter 3-7) and one chapter that reviews charophyte systematics (Chapter 2). Chapter 3 presents my findings in a phylogenetic study of the conjugating green algae based on three organellar genes. This study was designed to provide phylogenetic context for as many of the genera as could be obtained from culture collections and isolations from the wild. Based on these data, a number of observations were made and three lineages were investigated in greater detail.

The first is the filamentous Desmidiaceae (Chapters 4 and 5). It was found that some genera were polyphyletic and that most filamentous desmids formed a single lineage within the family. Because of the known differences in cell division, I investigated cell division patterns in greater detail in the filamentous Desmidiaceae (Chapter 4) and then investigated other aspects of their structure including cell and chloroplast shape. These characteristics were used in a systematic treatment of the filamentous genera where I propose moving two species to different genera and created one new generic name (Chapter 5).

The second case study investigated cryptic diversity in the Gonatozygaceae. Previous phylogenetic studies suggested a close relationship between *Gonatozygon* (Desmidiales) and *Roya* (Zygnematales). I also found in Chapter 2, a surprising degree of sequence diversity among structurally similar strains of *Gonatozygon*. To further investigate this, I increased taxon sampling in the Gonatozygaceae and investigated structural characteristics among the strains. The Gonatozygaceae are an excellent model for microbial diversity because, like many other unicellular organisms, they are structurally simple. Chapter 6 investigates the phyletic diversity within the family in an attempt to determine if the structural simplicity of the cells results in an underestimation of the species diversity. If so, I hoped to determine which characteristics distinguished lineages so that it might be possible to predict to which clade (or species) structurally similar organisms from the wild may belong.

The third case study investigated the phylogenetic position of *Triploceras gracile*. This genus is unique among the Desmidiaceae because some portions of the cell are radially symmetric while others are bilaterally symmetric. This character is often used in systematics of conjugating green algae and such transitions are considered important evolutionary events in the evolution of animals. In chapter 2, I found that *Triploceras* was embedded in a clade of *Micrasterias*. This placement was inconsistent with that reported by Gontcharov et al. (2003) based on SSU data. This raised the possibility of a complex evolutionary history. To investigate the evolutionary history of *Triploceras*, I sampled three, independent nuclear genes from several species of Desmidiaceae. The results of this investigation are presented in Chapter 7.

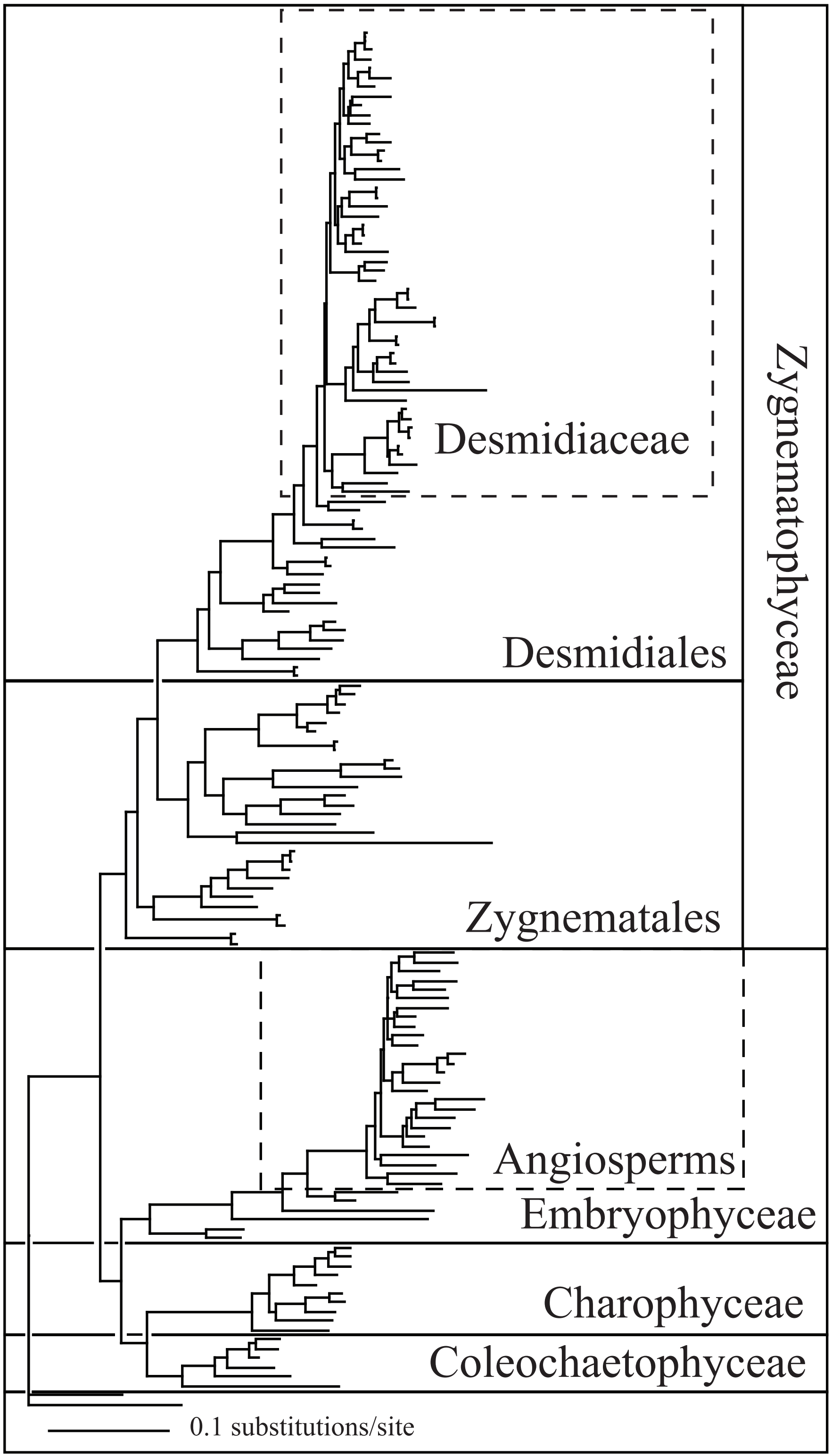

Figure 1.1 Maximum likelihood phylogeny of charophytes based on *rbcL*. Branchlengths are proportional. Note that the branchlengths in the family Desmidiaceae are comparable to those of all angiosperms (dashed boxes). Basal angiosperms and monocots were included in the analysis.

Chapter 2. In the shadow of giants; systematics of the charophyte green algae

Abstract

Charophyte green algae are those organisms most closely related to land plants. The group has at least five major lineages the Charophyceae, Coleochaetophyceae, Zygnematophyceae, Klebsormidiophyceae, Chlorokybophyceae and probably the Mesostigmatophyceae. These organisms are briefly introduced and their relative phylogenetic position discussed. Current systematic understanding of the groups is discussed as well as the potential role of genomic studies in the systematics of charophyte green algae. Genomic studies are beginning to elucidate the order of ancient branching events in the lineage, however, greater molecular and broader taxon sampling will be required to resolve some relationships. In addition to deep nodes, molecular phylogenetic investigations of populations and species of all the lineages are wanting. Continued investigation and greater sampling will provide more insight into the evolution of these organisms and early land plant evolution.

Introduction

Green algae are one of the most diverse groups of organisms on earth both structurally and in terms of number of described species. They occupy almost every habitat and are the algal relatives of the one of the most species-rich lineages, the land plants. Although land plants evolved from green algae, there are major diversifications in the green algae that preceded the invasion of land and radiation of embryophytes. One major distinction is between the Chlorophyta and the Charophyta (Charophyceae *sensu*

Mattox and Stewart, 1984). Land plants are members of the Charophyta and their closest algal relatives are here called the charophytes. The charophytes are only a small part of total green algal diversity, but their evolutionary history gives direct insight into the evolution of plants. Nomenclature used here follows Lewis and McCourt (2004; see Table 2.1). In this paper, 'charophyte' refers to those algae most closely related to land plants (not just the Charophyceae *sensu stricto*). When referring to Charophyceae *sensu stricto*, I use either Characeae or their common name, stoneworts.

Historical perspective

The charophytes, in the sense discussed here, have been recognized as a group only since the 1980s. They include their namesake Characeae (Charophyceae *sensu stricto*) and a collection of seemingly disparate lineages: the Coleochaetophyceae, the conjugating green algae (Zygnematophyceae), the Klebsormidiophyceae, *Chlorokybus atmophyticus* Geitler (Chlorokybophyceae) and, probably, *Mesostigma* (Mesostigmatophyceae). Some charophytes are quite large, such as members of the Characeae, and have been known for several hundreds of years: Linnaeus named some species of *Chara,* which had previously been described as aquatic forms of *Equisetum* (Wood and Imahori, 1965); others, such as *Spirogyra*, were among the first algae discovered by means of Van Leeuwenhoek's microscopes in the late 17th century (Leeuwenhoek, 1674). Even the relationship between green algae and land plants had long been supposed as implied by discussions of microscopic vegetables (Ingenhousz, 1779). Bower (1908) even described the plant-like characteristics of many algae, including some today recognized as charophytes. It was not until the latter part of the 20th century that science and technology (in this case electron microscopy) converged to provide evidence for the relationships among green algae and their affinity to land plants. The groups currently thought to belong to the charophyte lineage, with the exception of the polemic *Mesostigma viride* Lauterborn, were recognized by Mattox and Stewart (1984) in their systematic treatment of the green algae based on comparative cytology.

Charophyte systematics, however, did not begin in the 20th century. Members of these lineages were known in the 19th century and earlier. Two groups in particular, the Characeae and the conjugating green algae (Zygnematophyceae), include several hundreds or thousands of named species, respectively. Both of these have a long history of independent systematic investigation and several monographs are dedicated to their taxonomy and distribution. Pringsheim (1860) investigated *Coleochaete* and contemporary authors have critically studied this genus and described new species (Szymańska, 1989; Cimino and Delwiche, 2002; Delwiche et al., 2002). All the charophytes are commonly (and historically) included in local florulas, though rarely as a group unto themselves. Even today, valuable systematic data are often published as part of local or regional florulas. Systematic investigation, therefore, proceeds on many fronts: higher-level classification of the lineages and families as well as the population and species levels and molecular, genomic, cytological and morphological methods are used.

Table 2.1. Classification of the Charophyta based on Lewis and McCourt (2004)

Kingdom Viridiplantae

Division Chlorophyta

Division Charophyta

- Class Mesostigmatophyeae *sensu stricto*
- Class Chlorokybophyceae
- Class Klebsormidiophyceae
- Class Zygnematophyceae
  - Order Desmidiales
  - Order Zygnematales
- Class Coleochaetophyceae
- Class Charophyceae *sensu* G. M. Smith
- Class Embryophyceae [1]

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[1] This suffix is derived from the correct placement of embryophytes among charophytes, however, some embryophytologists may prefer a different rank or suffix.

Why should one study the systematics of charophytes?

The charophyte green algae hold a unique phylogenetic position as the closest extant relatives of terrestrial plants (in fact, embryophytes could be more correctly treated as a specialized charophyte lineage). Understanding of relationships within and among these lineages continues to provide insight into the evolution of land plants and their occupation of terrestrial habitats. Charophytes are also of systematic interest because they inhabit environments that are greatly affected by humans. Charophytes are primarily freshwater organisms (although a few occur in brackish pools) and many charophytes are most common in oligotrophic waters. These habitats are particularly impacted by human activities. There is a great need for monitoring and investigation of freshwater biodiversity, as estimates of the rate of extinction among freshwater organisms is very high (Leidy and Moyle, 1998; Watanabe, 2005). Few data are available on the conservation status of most charophycean taxa. Available data on Characeae and conjugating green algae indicate that local extinctions have occurred and global extinction may be likely. Few countries (including the United States) have biotic inventories that would reveal local extinctions, so the real loss of global charophyte biodiversity is unknown. Those countries with biotic inventories indicate that many charophyte taxa are threatened, endangered or already locally extinct (Krause, 1984; Siemińska, 1986; Stewart and Church, 1992; Adam, 2004; Németh, 2005; Watanabe, 2005). Charophyte systematics must continue with some haste if we are to record the global diversity and natural distribution of these important organisms.

Which green algae belong to the Charophyta?

The charophytes constitute one of the two primary lineages of green algae and are distinguished from their relatives, the chlorophytes, by a number of distinct if not immediately obvious features. Mattox and Stewart (1984) separated the charophyte algae from other green algae based on the presence of a multilayer structure at the base of the two flagella which insert subapically on the asymmetric flagellate cells. It is now known that multilayer structures occur in other groups of algae besides the charophytes, but the subapical insertion of two similar flagella is distinctive to the group. The Zygnematophyceae lack flagella and were included in the group because of their persistent mitotic spindle (Mattox and Stewart, 1984). Other characteristics were later discovered that indicated that the conjugating green algae are closely related to other charophytes, but these are not diagnostic of all charophytes. It is important to note that the charophytes are only a monophyletic group if land plants are considered among their ranks (Bhattacharya et al., 1998; Karol et al., 2001).

The classification provided by Lewis and McCourt (2004) assigns class status to the major lineages of charophytes, including the embryophytes (land plants). Higher-level nomenclature is still a source of disagreement among systematists, as are the relationships among the charophyte lineages. However, most authors do agree that the charophyte algae are different from other green algae (Chlorophyta), that they are closely related to land plants, and that most of the taxa proposed by Mattox and Stewart (1984) belong to this group. The inclusion of *Mesostigma viride* is less certain but seems very likely (see discussion below). The placement of embryophytes as a monophyletic lineage, deeply embedded within the charophytes, is not controversial, but the nomenclatural implications of this biological fact are highly so.

The number of charophyte species reported in the literature is not known with certainty. The best estimates are: Mesostigmatophyceae, 2; Chlorokybophyceae, 1 (Geitler, 1942b); Klebsormidiophyceae, 19 (Ettl and Gärtner, 1995; Lokhorst, 1996); Zygnematophyceae, 4,000-13,000 (Gerrath, 1993; Hoshaw et al. 1990); Coleochaetophyceae, 22 (Thompson, 1969; Szymańska, 2003); Charophyceae, 395 (Wood and Imahori, 1965). These estimates should be seen as minimum, provisional, good faith estimates taken from the literature, and should be interpreted with caution.

Species estimates among charophytes are difficult: inclusion of certain genera in the group is not certain and estimates are strongly biased by the treatment of varieties, particularly among the stoneworts and conjugating green algae. The total diversity of all other charophytes falls well within the uncertainty of estimates for the conjugating green algae alone. That said, estimates for the number of conjugating green algae range from about 3000 desmids (Gerrath, 1993) and 800 filamentous Zygnematales (Kadlubowska, 1984) to 10,000 -12,000 placoderm desmids excluding the filamentous and unicellular Zygnematales (Hoshaw et al., 1990). Differences in these estimates are due in part to the inaccessibility of the relevant literature: no one is certain how many taxa have been described, much less how many are synonymous. Another major factor in the variability of estimates is the treatment of varieties. Taxonomists of the Desmidiaceae (Zygnematophyceae) continue to use subspecies, varieties and forms, a practice abandoned in many other algal groups. This means that any one species name can have as many as several dozen subspecific taxa associated with it. The relationship between a desmid "species" and a biological species is not clear. If the varieties of conjugating green algae were treated as species, their number would likely approach the 12,000 species estimate of Hoshaw et al. (1990). Estimates of the number of species of charophytes are consequently subject to considerable interpretation.

It might be expected that with the number of papers dedicated to, in particular, the taxonomy of the conjugating green algae, there would be a clear understanding of their diversity or at least the number of species on earth. Unfortunately, information on their numbers and distribution is limited by the geographic location of investigators, accessibility of study sites, and time available for investigation. A limitation unique to the charophytes, to a greater or lesser degree, includes a tendency for some charophyte species to be overlooked in general floristic studies: *Coleochaete* grows attached to substrata, such as rocks and aquatic plants, which are frequently not collected; many desmids are benthic and do not appear in plankton studies; and smaller species of conjugating green algae and *Klebsormidium* are often mistaken for chlorophytes or xanthophytes in floristic surveys. As with many microscopic taxa, apparent distributions may be primarily a function of the geography of the investigators. It is also important to note that estimates only reflect the number of described taxa; the actual number of living charophytes in the world could be much greater. Vast portions of the world have not been investigated and new species are frequently described from even the best studied regions (e.g. Coesel, 2002; Szymanska, 2003).

General systematic studies of charophytes

Since the time the charophytes were formally recognized as a distinct group of green algae, few studies have investigated the relationships among these disparate lineages. Many of these studies have focused on two important questions: which taxon is most closely related to land plants and how might the ancestor of the charophytes (and possibly all green algae) have appeared? If these questions could be confidently answered, one could use the characteristics of the extant charophytes to make inferences about the evolution of land plants and charophyte algae. Systematic investigation, particularly molecular systematics, is necessary because the fossil record of these early diverging lineages is very poor; only the stoneworts (Charophyceae *sensu stricto*) have a well documented fossil record (Feist and Grambast-Fessard, 1990). Few molecular systematic investigations have focused on the relationships within the charophyte lineages.

Nearly every lineage of charophytes, or combination of lineages has been proposed as the most closely related to land plants at one time or another (Huss and Kranz, 1995; Kranz et al., 1995; Karol et al., 2001; Turmel et al., 2002c; Turmel et al., 2002a). However, most studies have very limited molecular or taxonomic sampling or both. Lemieux et al. (2000), based on a dataset of 53 genes and eight taxa, recovered *Mesostigma viride* at the base of the Viridiplantae clade. Other comparable phylogenies based on fewer genes support the placement of *Mesostigma* as the basal most lineage of the Charophyta (Bhattacharya et al., 1998; Karol et al., 2001). In a study using 72 mitochondrial genes and six taxa, Turmel et al. (2003) found a sister relationship between stoneworts and embryophytes. One molecular study (Karol et al., 2001) exhibits both substantial molecular sampling (4 genes) and a broad taxon sampling (40 taxa). This study found the stoneworts sister to land plants and the remaining lineages to be a paraphyletic assemblage with *Mesostigma* the earliest diverging member of the group

(Figure 5.2.1). The relationship between stoneworts and land plants was strongly supported, as was the placement of *Mesostigma* as the basal most charophyte, although the relationships among the other lineages of the charophytes received less support (Karol et al., 2001). It is worth noting, however, that several studies (particularly those using rDNA data) have found the stoneworts to be very distantly related to the land plants and, generally, suggest that the lineage most closely related to land plants is one or an assemblage of the other charophyte classes (Huss and Kranz, 1995; Kranz et al., 1995; Turmel et al., 2002c; Peterson et al., 2003). One study based on 76 chloroplast genes found the conjugating green algae (Zygnematophyceae) to be the sister lineage to land plants with stoneworts being only distantly related (Turmel et al., 2006). Evolution of the charophyte lineages is not completely understood.

Systematics of the charophyte lineages

Mesostigma

The earliest diverging lineage of the Charophyta may be *Mesostigma viride* Lauterborn (Figure 2.2A). This organism was originally classified among the prasinophyte green algae (scaly unicellular flagellates). On the basis of root configuration, Melkonian (1989) noted the affinity of *M. viride* to the charophytes. This was confirmed by analyses of actin sequences (Bhattacharya et al., 1998) that indicated *Mesostigma* is the basal most lineage of the Charophyta. *Mesostigma viride* is unicellular and covered in minute scales. Cells are circular and compressed with a concave inner surface (Melkonian, 1989). Unlike other charophytes, *Mesostigma viride* has two multilayer structures (MLSs) and an eyespot (Rogers et al., 1981). *Mesostigma viride* is known only from freshwater habitats, and has been infrequently reported from the wild.

Although its phylogenetic position is not known with certainty, a number of studies suggest that *Mesostigma viride* is either very ancient or very divergent (probably both) compared to other green algae: it has an unusual compliment of photosynthetic pigments (Yoshii et al., 2003; Stabenau and Winkler, 2005) and Stabenau and Winkler (2005) suggest that the microbodies found in *Mesostigma* may be ancestral to leaf peroxisomes and glyoxysomes since it has enzymes normally associated with both.

Some molecular phylogenetic studies place *Mesostigma* at the base of the Charophyta (Bhattacharya et al., 1998; Marin and Melkonian, 1999; Karol et al., 2001) while others find it to be sister to both the charophytes and chlorophytes (Lemieux et al., 2000; Turmel et al., 2002c; Turmel et al., 2002a). Evidence in favor of including *Mesostigma* in the Charophyta is mounting: *Mesostigma*, the other charophytes and land plants seem to share a unique duplication of the *GapA* /*GapB* gene (Peterson et al., 2006). Regardless of its position, *Mesostigma* is critical to our understanding of evolution of the green algae. At least one other species of *Mesostigma* has been described (*M. grande* Korshikov), but this species is not available in culture and, consequently, has not been thoroughly investigated.

Chlorokybus atmophyticus

Chlorokybus atmophyticus Geitler is the sole known representative of its lineage, the Chlorokybophyceae. The species was discovered growing among mosses in a park in Vienna, Austria (Geitler, 1942b). It is unique among the charophyte algae, with the possible exception of some conjugating green algae, in its sarcinoid growth habit, i.e. it grows as small packets of cells enveloped in a common mucilaginous matrix (Figure

2.2B). Its cell division, as well as production of autospores and swarmers, has been documented (Geitler, 1942a; Rieth, 1972). Sexual reproduction has not been reported. Zoospores of *C. atmophyticus* have a single multilayer structure and two subapical flagella that are covered in scales (Rieth, 1972; Rogers et al., 1980), a structure that is consistent with other flagellate charophyte cells. Although its distribution is unknown, it has been found in subaerial habitats in Europe and Russia (Geitler, 1942b; Rieth, 1972; Ettl and Gärtner, 1995).

Many molecular studies place *Chlorokybus atmophyticus* near the base of the Charophyta (Bhattacharya and Medlin, 1998; Karol et al., 2001). Phylogenetic analyses of 18S rDNA and chloroplast *rbc*L sequences suggests that *Spirotaenia* (normally considered to be a member of the Zygnematophyceae) may form a monophyletic group with *Chlorokybus* (Gontcharov and Melkonian, 2004). This relationship is certainly unexpected since *Spirotaenia* is known to lack flagella and to conjugate during sexual reproduction, characteristics typical of the conjugating green algae. The analysis showed only moderate statistical support for the group and could not rule out other placements of *Spirotaenia*. However, those data did reject a sister relationship between *Spirotaenia* and the conjugating green algae. The relationships of *Chlorokybus* and *Spirotaenia* to other charophyte algae certainly merit further investigation.

Klebsormidiophyceae

The Klebsormidiophyceae, particularly *Klebsormidium* spp. (Figure 2.2C), are among the most ubiquitous charophytes. The two genera most commonly included in the group (*Klebsormidium* and *Entransia*) are typically unbranched filamentous algae that reproduce by fragmentation of the filaments and the release of (presumably) biflagellate zoospores (Lockhorst, 1996; Cook, 2004a). Although structures consistent with zoosporangia were observed in *Entransia fimbriata* Hughes, no flagellate cells were observed (Cook, 2004). *Klebsormidium* typically has a single parietal chloroplast that partly encircles the cell with a single, embedded pyrenoid. Filaments are sometimes attached by holdfasts or interrupted by "H" pieces (Lockhorst, 1996). *Entransia* has one or two parietal chloroplasts that are deeply, irregularly lobed (see Cook, 2004, for discussion of morphology). Inclusion of other genera, such as *Stichococcus* and *Raphidonema* remains uncertain; some species of *Stichococcus* have been transferred to *Klebsormidium* (Ettl and Gärtner, 1995) while others clearly belong to the Chlorophyta (Lewis and Lewis, 2005).

The genus name *Klebsormidium* was created to accommodate *Hormidium sensu* Klebs. The taxon *Hormidium* was abandoned because of synonymy and general confusion (Silva et al., 1972). Since that time, a total of about seventeen species have been described or transferred to the genus. Taxonomy of the genus is based on morphological characters, such as: filament width, cell wall surface characteristics, and chloroplast shape (Lokhorst, 1996). *Klebsormidium* species are common in freshwater habitats but also occur in many subaerial habitats including desert crusts, urban walls, and freshwater seeps (Lewis and Fletchner, 2002; Johansen et al., 2004). *Entransia* is clearly distinct from *Klebsormidium,* seems to be rare and occurs infrequently in ponds, bogs, or seeps (Hughes, 1948; Prescott, 1966; Cook, 2004a).

Entransia was originally thought to belong to the conjugating green algae (Zygnematophyceae), although it is not known to conjugate. Only one of the two species has been investigated, *E. fimbriata*, which is thought to reproduce by zoospores (Cook, 2004a). Sexual reproduction has not been reported. Molecular phylogenetic investigations by McCourt et al. (2000) placed *E. fimbriata* outside the conjugating green algae and Karol et al. (2001) found it to be most closely related to *Klebsormidium*, a relationship supported by ultrastructural data (Cook, 2004a). Very few *Klebsormidium* isolates have been studied using molecular systematic methods (Novis, 2006) and more work certainly remains.

Zygnematophyceae

The Zygnematophyceae are the most species-rich clade of the Charophyta (excepting land plants). They are commonly referred to as the conjugating green algae because of their unusual mode of sexual reproduction by fusion of non-flagellate gametes. Zygnematophytes may be unicellular, colonial, or filamentous, depending on the species. Historically the filamentous species with smooth walls (Zygnemataceae) and the unicellular forms were treated separately, although they were thought to be closely related to one another long before the evolutionary process was understood (Ralfs, 1848). The current family-level classification of the conjugating green algae is based primarily on differences in cell wall structure (Mix, 1972). The class is often divided into two orders, the Zygnematales and the Desmidiales, with two and four families, respectively. The species number some thousands, with the majority belonging to the Desmidiaceae (Gerrath, 1993).

The placement of the Zygnematophyceae within the Charophyta is unclear. Most molecular studies place these algae sister to a clade with Coleochaetophyceae, stoneworts and land plants, or as part of a lineage sister to land plants and including other charophyte classes. Consistent with the hypothesis that the conjugating green algae are more closely related to land plants than *Klebsormidium* and *Chlorokybus* is the presence of a phragmoplast-like microtubule array in some Zygnematophyceae (Fowke and Pickett-Heaps, 1969a). Additionally, the Zygnematophyceae, Coleochaetophyceae, Characeae and land plants share similar cellulose synthesizing rosettes (Tsekos, 1999). However, one study using many chloroplast genes (but relatively few taxa) places the conjugating green algae sister to land plants (Turmel et al., 2006).

Molecular phylogenetic studies suggest that the two traditional families of the Zygnematales, the Zygnemataceae and the Mesotaeniaceae, are not monophyletic with respect to one another (McCourt et al., 1995; McCourt et al., 2000; Gontcharov et al., 2003). It also appears that many of the genera in the traditional family Mesotaeniaceae may not be monophyletic (Gontcharov et al., 2003, 2004). The order Zygnematales may not be monophyletic but rather consist of two lineages in paraphyly, one containing *Spirogyra* and *Sirogonium* and the other containing most of the remaining Zygnematales (McCourt et al., 2000; Gontcharov et al., 2003). The lineage that best corresponds to the classical Desmidiales (Figure 2.2D) almost certainly includes organisms formerly classified among the Mesotaeniaceae (Park et al., 1996; McCourt et al., 2000; Gontcharov et al., 2003). One study (Gontcharov and Melkonian, 2004) indicates that at least one genus classified among the conjugating green algae, *Spirotaenia*, may not be part of the main line of zygnematophyte evolution.

Taxonomy within the Zygnematophyceae has relied heavily on general morphology and fine structure, particularly cell wall structures (Desmidiaceae), or spore wall ornamentation (Zygnemataceae). Molecular phylogenetic methods may prove valuable for infrageneric phylogeny within the group, as indicated by the few studies that investigated species relationships (Denboh et al., 2001; Lee, 2001; Nam and Lee, 2001; Gontcharov and Melkonian, 2005). However, most of these studies did not test difficult relationships and different molecules and methods may be necessary for investigating closely related species.

Coleochaetophyceae

The Coleochaetophyceae are branched, filamentous epiphytes. Four genera, *Coleochaete*, *Chaetosphaeridium, Chaetotheke* and *Awadhiella*, are thought to belong to this group (Bourrelly, 1990; Delwiche et al., 2002). The organisms may be epiphytic, endophytic, or loosely attached to submerged vascular plants, Characeae or other suitable substrate, and are occasionally found free-floating. Sexual reproduction is oogamous in all species for which sexual reproduction has been described. Zoospores, meiospores and sperm are biflagellate with an MLS and lateral, subapical insertion of the flagella. Thalli are composed of compact or loosely branched filaments. *Chaetosphaeridium* is occasionally reported as unicellular, though this is true only in early developmental stages and mature plants form filaments (Thompson, 1969) albeit often with widely spaced cells. About 16 species of *Coleochaete* have been described (Szymańska, 2003), and at least 4 species of *Chaetosphaeridium* (Thompson, 1969). A single species of *Awahdiella* is known but it is extremely rare and its phylogenetic placement largely speculative. *Chaetotheke* is more common, but is difficult to recognize and it has received little study. A number of other genera have been classified among the

Coleochaetophyceae. Although many of these taxa can probably be referred to the Chlorophyta, others may legitimately belong to this lineage (Printz, 1964; Bourrelly, 1990).

The relationship between *Coleochaete* (Figure 2.2E) and *Chaetosphaeridium* has been assumed for some time, on the basis of their unusual sheathed hairs and similar chloroplast structure. Some phylogenetic analyses of 18S rDNA sequences indicate that the two may not be closely related, and place *Chaetosphaeridium* in a monophyletic group with *Mesostigma viride* at the base of the charophyte tree (Marin and Melkonian, 1999). My own analyses indicate that rDNA data provide weak support for such a placement, but analyses of *rbcL*, *atpB* and *nad5* consistently show the Coleochaetophyceae as a monophyletic group albeit often with modest bootstrap support (Karol et al., 2001; Cimino and Delwiche, 2002; Delwiche et al., 2002). This position is consistent with morphological and cytological characteristics (Karol et al., 2001; Delwiche et al., 2002). The phylogenetic placement of *Awahdiella* and *Chaetotheke* is unknown, although the filament structure, hairs and chloroplasts are similar to those of *Coleochaete* and *Chaetosphaeridium.*

The most recent treatment of *Chaetosphaeridium* is that of Thompson (1969), which is not a full monograph. The sparse information on *Chaetosphaeridium* is probably not because the organism is rare, but because it is easily overlooked and more difficult to isolate than other charophytes. *Coleochaete* has been the subject of more comprehensive systematic studies. The first monograph listed four species (Pringsheim, 1860) and a number of studies have revised and added to the work (Printz, 1964; Szymańska, 1989; Delwiche et al., 2002; Szymańska, 2003). Several new species were recently described including a previously unrecognized group characterized by incomplete envelopment of the zygote following fertilization, or incomplete “cortication” (Szymańska, 1988, 1989). Studies of endophytic strains akin to *C. nitellarum* Jost suggest that much of *Coleochaete* diversity remains undescribed (Cimino and Delwiche, 2002). Certainly further investigation will reveal still more species and, very likely, a greater structural diversity than is currently recognized.

Charophyceae sensu stricto (the stoneworts)

The stoneworts, or Charophyceae *sensu stricto,* are the most plant-like in appearance among the charophyte algae: they are macroscopic green algae with whorls of branches at nodes. Thalli may be monoecious or dioecious, depending on the species (Wood and Imahori, 1965; Corillion, 1972). All species are oogamous with motile sperm produced in complex antheridia. Oogonia and antheridia are surrounded by sterile jacket cells. Fertilized eggs (zygotes) develop a thick covering of sporopollenin. Zygotes and thalli may become impregnated with calcium carbonate (Wood and Imahori, 1965). These sporopollenin encrusted spores (called gyrogonites) are well preserved in the fossil record, which extends back in excess of 380 hundred million years (Feist and Grambast-Fessard, 1991). Six genera in a single family represent the extant Characeae. The two most common genera are *Chara* (Figure 2.2F) and *Nitella*. Fossil structural diversity is greater than extant diversity, however, and many families are known only from the fossil record. Taxonomy within the group has been greatly affected by Wood and Imahori (1965) who produced the most recent global monograph of the Characeae.

In their monograph, Wood and Imahori (1965) divide the family Characeae into two tribes, the Chareae (*Chara*, *Lamprothamnium*, *Nitellopsis*, and *Lychnothamnus*) and the Nitelleae (*Nitella* and *Tolypella*). Both seem to be monophyletic (Sanders et al., 2003), or the Nitellae may be paraphyletic (McCourt et al., 1996; McCourt et al., 1999). The genera, although represented by very few species in most analyses, seem to be monophyletic with the possible exception of *Chara*, which may include *Lamprothamnium*, based on 18S rDNA sequences and a broad taxon sampling within *Chara* (Meiers et al., 1999). Systematic investigation within the two largest genera, *Chara* and *Nitella*, is wanting. Molecular investigations of *Nitella* subgenus *Tieffallenia* suggest that some sections may be artificial and that mesospore membrane fine structure may be a valuable taxonomic characteristic, at least in subgenus *Tieffallenia* (Sakayama et al., 2004b; Sakayama et al., 2004a; Sakayama et al., 2005). Although Wood and Imahori (1965) reduced many described species to varieties, they also provided a list of the “species” described at that time, which they termed “microspecies.” The molecular investigations as well as other morphological studies seem to favor the “microspecies” concept (Wood and Imahori, 1965), particularly in the genus *Chara* (Corillion, 1972; Krause, 1997). Regardless of the treatment of varieties, there are probably a few hundred described, extant stoneworts.

Future directions and the role of genomics in charophyte systematics

Insights from published genomic data

Some charophyte organellar genomic data have been published, including the complete chloroplast genome of *Zygnema*, *Staurastrum* (Turmel et al., 2005), *Chaetosphaeridium* (Turmel et al., 2002a), *Chara* (Turmel et al., 2006) and *Mesostigma*

(Lemieux et al., 2000), as well as the mitochondrial genome of *Chara* (Turmel et al., 2003), *Chaetosphaeridium* (Turmel et al., 2002a) and *Mesostigma* (Turmel et al., 2002b). These studies provide insight into the evolution of chloroplast and mitochondrial genomes. In particular, complete organellar genomes provide information about gene content, gene order and trends in evolution of these genomes. The value of these sequences for systematic investigation will increase as more genome-scale data are collected from other charophyte algae. As of the beginning of 2006, no charophyte nuclear genome has been published (excluding embryophytes).

Besides organellar genomes, two expressed sequence tag (EST) surveys have been published. The first investigated the expressed mRNAs of members of the *Closterium peracerosum-stigosum-littorale* complex (Sekimoto et al., 2003) and related studies identified a pheromone that induces sexual cell division (Fukumoto et al., 2003a; Tsuchikane et al., 2003). The second survey sequenced more than 10,000 ESTs from *Mesostigma viride* and recovered transcripts of genes important for cellular processes such as translation and transcription, signaling and metabolism, though the majority have unknown functions (Simon et al., 2006). Such studies may provide the phylogenetic data necessary to resolve the branching order of the charophytes. However, it is important to remember the value of this information outside pure systematics. Genomic studies provide valuable information about the biology of these organisms and how their ancestors may have evolved to give rise to the complex metabolic pathways and gene families found in land plants. This information is critical to our understanding and interpretation of evolution within the charophytes as well as the origin and evolution of land plants.

Future systematic investigations

As noted above, the branching order of the deeper nodes (classes) remains uncertain. Molecular phylogenetic analyses and continued cytological observations may provide the data needed to answer these questions. Many published molecular datasets are very limited in character or taxon sampling, both of which affect inference of relationships. However, molecular investigations of currently available taxa may not be enough to resolve the branching order of the charophyte tree. Evolution within the lineages will remain uncertain until more taxa are available for investigation. Within the Zygnematophyceae, for instance, the known structural diversity has yet to be probed. Relationships among orders, families and genera of the conjugating green algae remain poorly resolved. Fewer than half of the known genera of Zygnematophyceae are available for molecular investigation. Molecular phylogenetic analyses of the Klebsomidiophyceae remain in their early stages and many relationships remain unclear in the Coleochaetophyceae and Characeae. Future studies may depend on a broader sampling of taxa as well as more sizable molecular datasets.

None of the charophyte lineages has been comprehensively surveyed by molecular methods. Species relationships remain poorly understood in all but the Coleochaetophyceae. Published work on the Characeae and Zygnematophyceae has only begun to address species relationships using molecular methods, and population level studies remain few. Although a number of outstanding morphological, mating and AFLP analyses have been published (Griffiin and Proctor, 1964; Grant and Proctor, 1972;

Meiers et al., 1999; Mannschreck et al., 2002), very few studies have addressed the structure of charophyte populations using molecular sequence techniques. How populations interact as well as the distribution of charophyte algae are generally unknown. Even though there are scattered reports for Characeae and Zygnematophyceae, little is known about global population status and local surveys suggest that many populations may be severely pressured (Krause, 1984; Siemińska, 1986; Stewart and Church, 1992; Németh, 2005; Watanabe, 2005).

Besides the ordering and documentation of the known species, systematists are also concerned with the discovery of new species. A number of extensive floristic studies exist, but as is often the case for widespread and taxonomically difficult taxa, these are strongly biased by geographic location of investigators and accessibility of study sites. The physically smaller species in particular have been poorly documented. It is difficult to imagine that *Chlorokybus* and *Klebsormidium* are represented by the relatively few described species particularly since *Klebsormidium* thrives in a wide range of habitats. A great many species are likely to be hidden in such unlikely places as university fountains and garden walls, as was the case for *Chlorokybus* (Geitler, 1942b).

Another strong bias in current charophyte systematics is the "microbial bias." Except for the Characeae, nearly all charophytes used in molecular phylogenetic studies have been cultured from the wild. This almost certainly introduces biases in the investigation analogous to those encountered when surveying Bacteria and Archea by culturing. As a complement to traditional culture-based methods, molecular sequence data provide an independent means of investigating diversity that eliminates the culture bias, though it introduces others. Molecular environmental studies of even well characterized habitats have received a lot of attention because many new lineages have been discovered (López-García et al., 2001; Venter et al., 2004). Relating these sequences to the organisms from which they came, however, is difficult. Not just new species, but new kinds of organisms with potentially different life histories and metabolic pathways may yet be found.

Systematic investigation of the charophyte green algae will continue to provide insight into the diversity and evolution of these exceptional organisms. Future studies will, hopefully, embrace new techniques and technologies and use them to answer fundamental systematic questions. Much remains to be investigated at all levels and in all charophyte lineages.

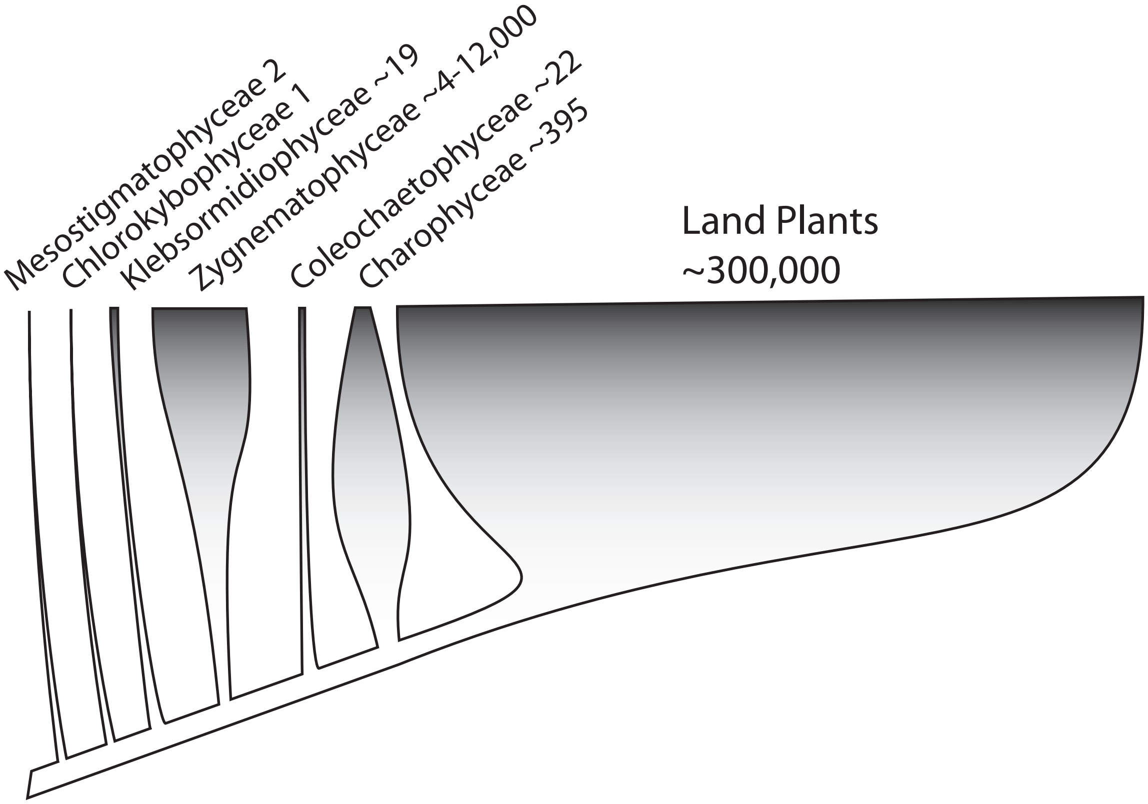


Figure 2.1. Phylogeny of charophytes showing species richness. Branching order of the lineages of Charophyta based on Karol et al. 2001. Line width at top of diagram is approximately proportional to the number of described species for the group. Charophyceae and the land plants are the only lineages for which there are ancient fossils. These fossils indicate that the Charophyceae were probably more diverse in the past than they are at present. For the other lineages of charophyte algae, their prehistoric diversity is almost completely unknown.

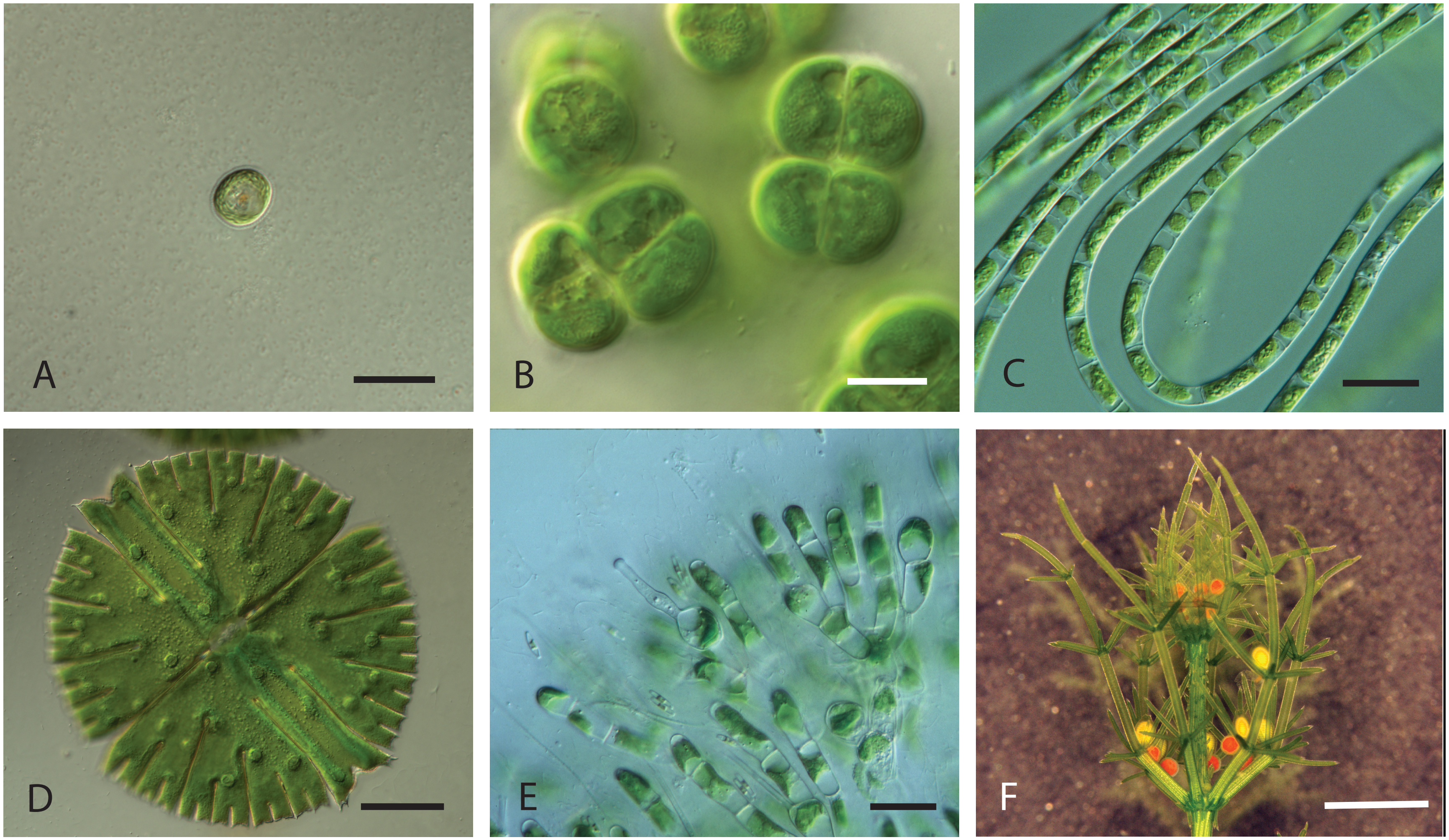


Figure 2.2. Light micrographs of representatives of the major lineages of the Charophyta. A. *Mesostigma viride*, scale bar = 20 um; B. *Chlorokybus atmophyticus*, scalebar = 10um; C. *Klebsormidium flaccidum*, scale bar = 25 um; *Micrasterias rotata*, scale bar = 50 um; E. *Coleochaete pulvinata*, scalebar = 25 um; F. *Chara tomentosa*, scale bar = 5 mm.

Chapter 3. Phylogeny of the conjugating green algae based on chloroplast and mitochondrial nucleotide sequence data

Abstract

The conjugating green algae are a lineage of charophyte green algae known for their structural diversity and unusual mode of sexual reproduction, conjugation. These algae are ubiquitous in freshwater environments, where they are often important primary producers, but few studies have investigated evolutionary relationships in a molecular systematic context. A 109 taxon dataset consisting of three gene fragments (two from the chloroplast and one from the mitochondrial genome) was used to estimate the phylogeny of the genera of the conjugating green algae. Maximum Likelihood, Parsimony and Bayesian Inference were used to estimate relationships from the 4047 alignable nucleotides. This study confirmed polyphyly of the Zygnemataceae and Mesotaeniaceae with respect to one another. The Peniaceae were found to be paraphyletic, and two genera traditionally classified among the Zygnematales appear to belong to the lineage that gave rise to the Desmidiales. Six genera, *Euastrum*, *Cosmarium, Cylindrocystis, Mesotaenium, Spondylosium* and *Staurodesmus,* were found to be polyphyletic. These findings have important implications for the evolution of structural characteristics in the group, and will require some taxonomic changes. More work will be required to delineate lineages of Zygnematales in particular, and to identify structural synapomorphies for some of the newly identified clades.

Introduction

Charophyte green algae constitute one of the two major lineages of green algae and include those most closely related to land plants. The charophyte algae are, therefore, critical to our understanding of evolution of land plants and their invasion of the terrestrial environment. They are also prominent members of the freshwater microflora, and in some systems make a major contribution to primary productivity. There are six primary clades of charophyte algae (Lewis and McCourt, 2004). Each of these lineages is thought to be ancient, and yet many are represented by very few (1-25) known species. However, one lineage, the conjugating green algae (Zygnematophyceae), contains the majority of charophyte taxonomic diversity, with several thousand named species. These organisms are remarkable among green algae for their diversity of cell wall structure and ornamentation. Additionally, members of this group have been important to the understanding of plant processes including cell wall morphogenesis and phytochrome signaling (Bock and Haupt 1961, Haupt 1959, 1960, Haupt and Bock 1962, Meindl 1993, Walczak et al. 1990, Winands and Wagner 1996). The conjugating green algae are among the most common of all freshwater algae, and members of the group can be found in a number of extreme habitats including acid bogs, alkaline streams, desert crusts, and snow. Although widely distributed, the group's diversity is poorly understood, and many phylogenetic relationships remain untested with molecular methods.

The conjugating green algae (zygnematophytes) include the traditional Zygnemataceae as well as the "saccoderm" and "placoderm" desmids (Mix 1972). These comprise a species-rich and structurally heterogeneous group clearly embedded within the charophyte green algae, and united by their unique mode of sexual reproduction, conjugation, and a complete absence of a flagellate stage in the lifecycle. Many species are difficult to identify, especially the smallest taxa, and some species are differentiated based on infrequently observed sexual reproductive characteristics.

Taxonomic concepts of the families and orders within the conjugating green algae are based primarily on ultrastructural characteristics. Mix (1972) classified the conjugating green algae in two orders and six families based on the structure of their cell walls. The order Zygnematales included conjugating green algae whose walls are smooth and whose primary wall is indehiscent. Species in this order were classified in two families, the Mesotaeniaceae (unicellular organisms) and the Zygnemataceae (filamentous organisms). The order Desmidiales included the remaining conjugating green algae whose walls are interrupted by sutures, pores, or both. The Desmidiales were classified in two suborders and four families: the family Desmidiaceae in the suborder Desmidiineae and the Closteriaceae, Gonatozygaceae and Peniaceae in the suborder Archidesmidiineae (Mix 1972). Zygnematales seem to be homogenous with respect to the construction of their cell walls, but their assignment to families based on growth habit (unicellular vs. filamentous) is artificial (Park et al. 1996, McCourt et al. 1995, McCourt et al. 2000, Gontcharov et al. 2003, 2004). Each of the families of the Desmidiales was characterized by a unique cell wall construction (Mix 1972)

While Mix's (1972) publication is the most recent widely accepted classification of the conjugating green algae, there have been a number of revisions proposed. Kouwets and Coesel (1984) proposed that the Gonatozygaceae be merged into the Peniaceae. Various authors have proposed the abandonment of certain higher groups because molecular data suggest that they are not monophyletic; these include the

Mesotaeniaceae and Zygnemataceae *sensu stricto* (McCourt et al. 1995, Park et al. 1996, McCourt et al. 2000, Gerrath 2003, Gontcharov et al. 2003, 2004) and the Archidesmidiineae (McCourt et al. 2000, Gontcharov et al. 2004).

The few published molecular phylogenetic studies, based on nuclear large-subunit ribosomal data, the chloroplast gene *rbcL*, or both, have confirmed the monophyly of the group (McCourt et al. 2000, Gontcharov et al. 2003, 2004), with the possible exception of *Spirotaenia* (Gontcharov and Melkonian 2004). These studies investigated the relationships of the families and genera, but the genera were in most cases represented by only one or a very few exemplar taxa. A relatively wide sampling of species within select genera was presented by Gontcharov et al. (2004), and Gontcharov and Melkonian (2005) used non-coding ITS1-ITS2 to test the relationships among *Staurastrum*-like species. Drummond et al. (2005) investigated the relationships among species of *Spirogyra* and *Sirogonium* and found these two genera to be distinct and each monophyletic, with the exception of *Spirogyra maxima* UTEX 2495, which was found to be embedded in a clade of *Sirogonium*.

Despite this progress, many generic relationships in the conjugating green algae remain untested or poorly resolved. The present study used a multi-gene dataset, including two newly developed molecular markers, and dense taxon representation to test the concepts of families and genera of the conjugating green algae. Because many genera are species rich and structurally diverse, it was not practical to exhaustively test the monophyly of all the genera, but the relatively fine-grained taxon sampling made it possible to identify some non-monophyletic genera, and confirm the likely monophyly of others.

Materials and Methods

Strains

Strains used in this study were obtained from public culture collections or isolated from the wild (see Table 3.1). Strains were maintained in liquid media; either Guillard's Woods Hole Medium, Bold's Basal Medium (Nichols 1973), or Bold's Basal Medium enriched with 20 $mL \cdot L^{-1}$ of soil extract. Cells grew in 25 mL flasks in a Percival growth chamber (Percival, Perry, IA) with a light: dark cycle of 16:8 hrs under Sylvania Cool White fluorescent lamps (Danvers, MA). Cells were observed at time of accessioning and extraction for contamination. All strains were identified morphologically with light microscopy; in several cases the diagnosis was different from the name attached to the strain in culture collection records (Table 3.1). Corrected names are provided in Table 3.1, with the culture name in brackets. Other sampled taxa have been assigned revised names as proposed by other authors. Synonomy is provided in parentheses in Table 3.1.

Table 3.1 Strains investigated and GenBank numbers

Taxon	Strain	*rbcL*	*psaA*	*coxIII*
Zygnematophyceae				
Desmidiales				
Desmidiaceae				
Actinotaenium (Penium) silvae-nigrae (Rabanus) Kouwets and Coesel	SVCK 295	**EF371323**	**EF371223**	**EF371116**
Actinotaenium cucurbita (Brébisson) Teiling ex Ruzicka & Pouzar	JH0383	----------	**EF371173**	**EF371066**
Actinotaenium curcurbitinum (Bisset) Teiling	ACOI 901	**EF371279**	**EF371172**	**EF371065**
Bambusina borreri (Ralfs) Cleve	JH0199	**EF371283**	**EF371177**	**EF371070**
Bambusina borreri (Ralfs) Cleve	JH0125	**EF371284**	**EF371178**	**EF371071**
Cosmarium botrytis (Meneghini) Ralfs	UTEX 301	**EF371288**	**EF371182**	**EF371075**
Cosmarium sp.	JH0041	**EF371290**	**EF371184**	**EF371077**
Cosmarium pseudoconnatum Nordstedt	JH0264	**EF371280**	**EF371174**	**EF371067**
Cosmarium melanosporum Archer	JH0011	**EF371289**	**EF371183**	**EF371076**
Cosmocladium saxonicum Hilse	ACOI 95	**EF371292**	**EF371186**	**EF371079**
Desmidium aptogonum var. *ehrenbergii* Kützing	JH0184	**EF371298**	**EF371192**	**EF371085**
Desmidium baileyi (Ralfs) Nordstedt	JH0228	**EF371299**	**EF371193**	**EF371086**
Desmidium swartzii Agardh ex Ralfs	JH0042	**EF371297**	**EF371191**	**EF371084**
Euastrum intermedium Gay	JH0159	**EF371302**	**EF371196**	**EF371089**
Euastrum crassum var. *michiganense* Prescott	JH0018	**EF371300**	**EF371194**	**EF371087**
Euastrum verrucosum Ehrenberg ex Ralfs	JH0068	**EF371301**	**EF371195**	**EF371088**
Haplotaenium (Pleurotaenium) minutum (Ralfs) Bando	SVCK 302	**EF371326**	**EF371227**	**EF371120**
Heimansia (Cosmocladium) pusillum (Hilse) Coesel	SVCK 428	**EF371291**	**EF371185**	**EF371078**
Hyalotheca dissiliens Brébisson ex Ralfs	SAG 384-2	AF203499	**EF371202**	**EF371095**
Hyalotheca mucosa (Mertens) Ehrenberg ex Ralfs	JH0055	**EF371305**	**EF371203**	**EF371096**
Hyalotheca mucosa (Mertens) Ehrenberg ex Ralfs	JH0063	**EF371306**	**EF371204**	**EF371097**
Micrasterias foliacea Bailey	NIES 297	**EF371311**	**EF371209**	**EF371102**
Micrasterias radiata Hassall	JH0064	**EF371313**	**EF371211**	**EF371104**
Micrasterias rotata (Greville) Ralfs	UTEX 1941	**EF371312**	**EF371210**	**EF371103**
Onychonema laeve var. *micracanthum* Nordstedt	JH0198	**EF371318**	**EF371218**	**EF371111**
Onychonema laeve var. *micracanthum* Nordstedt	JH0266	**EF371319**	**EF371219**	**EF371112**
Phymatodocis nordstedtiana Wolle	SAG 47.89	AJ553962	**EF371225**	**EF371118**
Phymatodocis nordstedtiana Wolle	JH0164	**EF371325**	**EF371226**	**EF371119**
Pleurotaenium baculoides (Roy & Bisset) Playfair	JH0008	**EF371327**	**EF371228**	**EF371121**
Pleurotaenium constrictum (Bailey) Wood	JH0135	**EF371328**	**EF371229**	**EF371122**
Pleurotaenium ehrenbergii f. *columellare* Irénée-Marie	JH0331	**EF371329**	**EF371230**	**EF371123**
Spondylosium tetragonum West	JH0175	**EF371304**	----------	----------
Spondylosium tetragonum West	JH0281	**EF371336**	**EF371239**	----------
Spondylosium tetragonum West [*Groenbladia undulata*]	SVCK 440	AF203498	**EF371201**	**EF371094**
Spondylosium pulchellum Archer	SVCK 365	AF203505	**EF371245**	**EF371137**
Spondylosium pulchrum (Bailey ex Ralfs) Archer	JH0269	**EF371341**	**EF371246**	**EF371138**
Staurastrum tetracerum (Kützing) Ralfs *[Arthrodesmus* sp.]	UTCC 348	**EF371282**	**EF371176**	**EF371069**

Staurastrum arctiscon (Ehrenberg) Lundell	JH0014	**EF371343**	**EF371248**	**EF371140**
Staurastrum arctiscon (Ehrenberg) Lundell	JH0070	**EF371346**	**EF371251**	**EF371143**
Staurastrum pseudosuecicum Prescott and Scott	JH0010	**EF371342**	**EF371247**	**EF371139**
Staurastrum polytrichum f. *biseriatum* Kaiser	JH0015	**EF371344**	**EF371249**	**EF371141**
Staurastrum gladiosum Turner	JH0132	**EF371347**	**EF371252**	**EF371144**
Staurstrum brevispinum Brébisson ex Ralfs	JH0180	**EF371349**	**EF371254**	**EF371146**
Staurastrum polymorphum Brébisson	JH0053	**EF371345**	**EF371250**	**EF371142**
Staurodesmus convergens (Ehrenberg) Teiling *[Arthrodesmus* sp.]	UTEX 2508	**EF371281**	**EF371175**	**EF371068**
Staurodesmus extensus (Anders.) Teiling	JH0386	**EF371317**	**EF371217**	**EF371110**
Staurodesmus mamillatus (Nordstedt) Teiling	JH0090	**EF371348**	**EF371253**	**EF371145**
Teilingia (Sphaerozosma) granulata (Roy and Bisset) Bourelly	SAG 39.83	**EF371335**	**EF371237**	**EF371130**
Teilingia granulata (Roy and Bisset) Bourelly	JH0140	**EF371351**	**EF371255**	**EF371148**
Teilingia granulata (Roy and Bisset) Bourelly	SAG 25.88	**EF371350**	**EF371256**	**EF371147**
Teilingia granulata [Sphaerozosma sp.] (Roy and Bisset) Bourelly	UTCC 284	AF203504	**EF371238**	**EF371131**
Tetmemorus brebissonii (Meneghini) Ralfs	SVCK 409	**EF371352**	**EF371257**	**EF371149**
Tetmemorus laevis (Kützing) Ralfs	SVCK 227	**EF371353**	**EF371258**	**EF371150**
Triploceras gracile Bailey	SAG 24.82	**EF371354**	**EF371259**	**EF371151**
Xanthidium antilopaeum (Bréb. Ex Meneghini) Kützing	JH0261	**EF371355**	**EF371261**	**EF371153**
Xanthidium hastiferum Turner	JH0054	**EF378638**	**EF371260**	**EF371152**
Peniaceae				
Penium cf. *didymocarpum* Lundell	JH0212	**EF371316**	**EF371216**	**EF371109**
Penium cylindrus (Ehrenberg) Brébisson ex Ralfs	ACOI 780	**EF371320**	**EF371220**	**EF371113**
Penium margaritaceum (Ehrenberg) Brébisson ex Ralfs	UTEX 600	**EF371321**	**EF371221**	**EF371114**
Penium margaritaceum (Ehrenberg) Brébisson ex Ralfs	ACOI 330	**EF371322**	**EF371222**	**EF371115**
Penium spirostriolatum Barker	SVCK 205	**EF371324**	**EF371224**	**EF371117**
Closteriaceae				
Closterium acerosum (Schrank) Ehrenberg ex Ralfs	UTEX 1075	**EF371285**	**EF371179**	**EF371072**
Closterium ehrenbergii var. *malinvernianum* (De Notaris) Rabenhorst	JH0013	**EF371286**	**EF371180**	**EF371073**
Closterium libellula Focke	JH0021	**EF371287**	**EF371181**	**EF371074**
Spinoclosterium cuspidatum (Bailey) Hirano	NIES 325	AJ553965	**EF371240**	**EF371132**
Gonatozygaceae				
Gonatozygon pilosum Wolle	ACOI 1096	**EF371303**	**EF371200**	**EF371093**
Gonatozygon kinahanii (Archer) Rabenhorst	ACOI 350	AJ553945	**EF371198**	**EF371091**
Gonatozygon brebissonii var. *laeve* (Hilse) West & West	JH0033	**EF371332**	**EF371233**	**EF371126**
Genicularia spirotaeniae de Bary	SAG 54.86	AJ553946	**EF371197**	**EF371090**
Gonatozygon monotaenium de Bary	UTEX 1253	U71438	**EF371199**	**EF371092**
Zygnematales				
Mesotaeniaceae				
Cylindrocystis brebissonii Meneghini	UTEX 1259	**EF371293**	**EF371187**	**EF371080**

Cylindrocystis crassa de Bary	ACOI 310	**EF371294**	**EF371188**	**EF371081**
Cylindrocystis sp.	UTEX 1925	**EF371295**	**EF371189**	**EF371082**
Cylindrocystis sp.	JH0038	**EF371296**	**EF371190**	**EF371083**
Mesotaenium caldariorum (Lagerheim) Hansgirg	UTEX 41	**EF371307**	**EF371205**	**EF371098**
Mesotaenium endlicharianum Nägeli	ACOI 451	**EF371308**	**EF371206**	**EF371099**
Mesotaenium kramstai Lemmermann	UTEX 1024	**EF371309**	**EF371207**	**EF371100**
Mesotaenium sp.	JH0031	**EF371310**	**EF371208**	**EF371101**
Netrium digitus (Ehrenberg) Itzigson & Rothe	UTEX 561	U38698	**EF371214**	**EF371107**
Netrium digitus var. *lamellosum* (Brébisson) Grönblad	UTEX 1255	**EF371315**	**EF371215**	**EF371108**
Roya anglica West	UTEX 934	AJ553963	**EF371231**	**EF371124**
Roya obtusa (Brébisson) West & West	SAG 168.80	**EF371331**	**EF371232**	**EF371125**
Zygnemataceae				
Mougeotia sp.	UTEX 758	AF408252	**EF371212**	**EF371105**
Mougeotia sp.	JH0040	**EF371314**	**EF371213**	**EF371106**
Mougeotia sp.	JH0304	**EF371364**	**EF371270**	**EF371163**
Sirogonium melanosporum (Randhawa) Transeau	ARL 700	L13484	**EF371234**	**EF371127**
Sirogonium sp.	JH0082	DQ015929	**EF371236**	**EF371129**
Sirogonium sticticum (Engler) Kützing	UTEX 1985	DQ015924	**EF371235**	**EF371128**
Spirogyra grevilleana (Hassall) Kützing	UTEX 477	DQ015938	**EF371243**	**EF371135**
Spirogyra condensata (Vaucher) Kützing	UTEX 1744	DQ015936	**EF371242**	**EF371134**
Spirogyra gracilis (Hassall) Kützing	UTEX 1743	DQ015937	**EF371241**	**EF371133**
Spirogyra maxima (Hassall) Wittrock	UTEX 2495	DQ15941	**EF371244**	**EF371136**
Zygnema circumcarinatum Czurda	UTEX 42	**EF371356**	----------	**EF371154**
Zygnema cylindricum Transeau	SAG 689-2	**EF371357**	**EF371262**	**EF371155**
Zygnema peliosporum Wittrock	UTEX 45	U38701	**EF371263**	**EF371156**
Zygnema sp.	JH0039	**EF371360**	**EF371265**	**EF371158**
Zygnema sp.	JH0087	**EF371362**	**EF371267**	**EF371160**
Zygnema sp.	JH0049	**EF371361**	**EF371266**	**EF371159**
Zygnema sp.	JH0007	**EF371359**	**EF371264**	**EF371157**
Zygnemopsis minuta Randhawa	ACOI 60	**EF371363**	**EF371268**	**EF371161**
Zygogonium tunetanum Gauthier-Lièvre	UTCC 136	AF203509	**EF371269**	**EF371162**
Outgroup				
Coleocheatales				
Coleochaete scutata Brébisson	SAG 3.9	AY082324.1	**EF371273**	**EF371166**
Coleochaete nitellarum Jost	UTEX 1261	AY082325.1	**EF371275**	**EF371168**
Coleochaete divergens Pringsheim	300d1	AY082324.1	**EF371272**	**EF371165**
Coleochaete pulvinata Braun ex Kützing	57b6	AY082307.1	**EF371274**	**EF371167**
Coleochaete sieminskiana Szymanska	10d1	AF408791.1	**EF371277**	**EF371170**
Chaetosphaeridium globosum (Nordstedt) Klebahn	SAG 26.98	AF408792.1	**EF371276**	**EF371169**
Chlorokybales				
Chlorokybus atmophyiticus Geitler	UTEX	AF408805.1	**EF371271**	**EF371164**

	2591			
Mesostignematales				
Mesostigma viride Lauterborn	SAG 50-1	AF408806.1	**EF371278**	**EF371171**

Notes: ACOI, Coimbra Collection of Algae; NIES, National Institute for Environmental Studies; SAG, Sammlung von Algenkulturen der Universität Göttingen; SVCK, Sammlung von Conjugaten-Kulturen; UTCC, University of Toronto Culture Collection of Algae and Cyanobacteria; UTEX, Culture Collection of Algae at University of Texas. Vouchers of strains beginning with JH are available from the authors. Sequences generated for this study are in bold.

Table 3.2. Primers used for PCR amplification of fragments

psaA 2100R	GGCAATTCCACCCAGAAGG
psaA IF	TTCCTTTGCCTCATGAATTC
psaA 569F	GGGCTGGTCATCAAATTCATGTGTC
*cox*III 5'ZYG	GGCGGTGTTATGTACATGCA
*cox*III 3'G	CAGCTGCTTCAAAGCCAAAGTGA

DNA extraction and fragment amplification

DNA was extracted using the Nucleon Phytopure DNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's protocol with an additional chloroform separation. Three genes were chosen (chloroplast ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) and photosystem I P700 chlorophyll A apoprotein A1 (*psa*A) and mitochondrial cytochrome oxidase subunit III (*cox*III)) to infer the phylogenetic relationships. Portions of the chloroplast gene rbcL were amplified using published primers (McCourt et al. 2000). Other gene fragments were amplified by PCR using the primers listed in Table 3.2. All genes were amplified on a Whatman Biometra (Göttingen, Germany) T-Gradient thermal cycler. These primers, and other taxon specific internal primers, were used to sequence the fragments of the *rbcL, psaA* and *cox*III genes, on an Applied Biosystems AB 3100 or AB 3730 capillary sequencer using Big Dye Terminator sequencing chemistry (Applied Biosystems, Foster City, California). Fragments were sequenced in both directions and assembled using Sequencher 4.2 (GeneCodes, Ann Arbor, MI).

Phylogenetic analyses

Sequences were aligned using MacClade 4.07 (Maddison and Maddison 2000), and phylogenetic analyses performed in PAUP* version 4b.10 (Swofford 2003), phyML (Guindon and Gascuel 2003) and MrBayes 3.1 (Huelsenbeck and Ronquist 2000). Portions of the alignment were excluded from the analyses because of indels or missing data: positions 1753-1758, 1969-1992, 2953-2958, and 3226-3228 of the multi-gene alignment. Genes were aligned individually and analyzed under Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) (data not shown). Homogeneity of base frequency among taxa was tested using a X^2 test as implemented in in PAUP*. Each of the genes was found to be homogenous among taxa (data not shown). Estimated topologies were generally consistent among the three genes, although none confidently resolved deeper nodes (data not shown).

Gene fragments were concatenated into a single dataset. This concatenated dataset was analyzed with and without third codon position. Phylogenetic trees were estimated under the MP and ML criteria in PAUP* as well as using Bayesian Inference (BI) in MrBayes. Non-parametric bootstrap (BS) under MP and ML and posterior probability (PP) in BI were used as estimates of support for taxon bipartitions. Ten random sequence addition replications and tree bisection-reconnection (TBR) branch swapping were used during the heuristic search under the Parsimony criterion; support was estimated from 500 BS replications with TBR branch swapping and ten random addition sequence repetitions. Under the Maximum Likelihood criterion I searched for the best tree using three random addition sequence replicates. Support was estimated using 100 BS replicates with a single random addition sequence and TBR branch swapping. I used the GTR + I + Γ model, with all parameters estimated from the data as recommended by MrModeltest 2.2 (Nylander 2004). BS analyses were performed in PhyML.

Two independent analyses using Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) methods in MrBayes were used with four chains sampled for four million generations to estimate topology, parameter values, and the posterior probabilities of the taxon bipartitions. The chains were sampled every one hundred generations and the first two thousand samples discarded as burn-in. Both analyses converged on similar topologies. Individual genes were analyzed similarly but with 100 MP and ML BS pseudoreplicates and two million generations under BI.

Because the best trees were inconsistent with current taxonomic concepts, a number of alternative topologies were explicitly tested. To reduce computation time, the 109 taxon dataset was sampled to include 55 taxa, including all Zygnematales and a subset of the Desmidiaceae. Tree files were edited manually to constrain regions of interest in the tree. These files were then used as constraints and the ML tree given those constraints were estimated. To save time when estimating the most likely alternative trees, the outgroup genus *Coleochaete* was constrained as monophyletic, as were *Zygnema* spp.. The positions of all other taxa were allowed to vary. These topologies were tested using the Approximately Unbiased (AU) test (Shimodaira, 2002), in Consel v. 0.1h (Shimodaira and Hasegawa, 2001).

Results

Taxa

When preparing for DNA extraction, it was found that several strains from culture collections were contaminated or misidentified. Contaminated strains were excluded from the study and those misidentified, when possible, were assigned to the correct genus and species. This resulted in the absence in this analysis of some genera and several strains that were included in previous studies. No strain consistent with the morphological description of *Sphaerozosma* or *Groenbladia* was available for this study (see discussion). *Arthrodesmus* sp. UTCC 348 is here identified as *Staurastrum tetracerum*.

Multigene phylogeny

This study included more taxa (109) and more nucleotide characters (4047) than any previous analysis of the conjugating green algae. In broad terms the analyses were consistent with those based on *rbcL* and SSU rDNA. Use of multiple genes from two organellar genomes permitted relatively confident inference of branching order of the major lineages of the Desmidiales. Early branching events in the Zygnematales remains poorly resolved. Topologies from analysis of individual genes were consistent with that of the combined dataset although no individual gene tree resolved deeper nodes with confidence (data not shown). By combining the genes into a single dataset, I wase able to more confidently estimate many relationships among the taxa.

Although this study was not designed to test the monophyly of the conjugating green algae and excluded some taxa that are important to that question (i.e. Klebsormidiales and *Spirotaenia* spp.), the conjugating green algae included in this study formed a monophyletic clade with high support (100/100/1.0; MP/ML/PP). *Spirogyra* and *Sirogonium* spp. formed a monophyletic clade (100/100/1.0) that was the sister taxon to the remainder of the conjugating green algae, diverging before the separation of the Desmidiales and the other Zygnematales, however *Spirogyra* was not monophyletic with respect to *Sirogonium,* even with the assumption that *Spirogyra maxima* is a *Sirogonium* sp. (Figure 3.1). However, *Spirogyra* and *Sirogonium* together formed a monophyletic group (100/100/1.0).

A second major lineage of zygnematalean taxa, including most of the remaining genera of the *Zygnematales,* except *Netrium* spp. and *Roya* spp., was resolved, but with little support (-/73/1.0). Within this lineage, several strains of *Zygnema* form a well-supported (100/100/1.0) monophyletic clade. One species, *Zygnema circumcarinatum* shares 99.7% sequence identity with *Zygogonium tunetanum*. The significance of this relationship is unclear because neither strain could be confidently identified (see discussion). Although some strains of *Mesotaenium* seem to be very divergent, one strain, *M. caldariorum*, was consistently resolved as sister to *Mougeotia* (97/100/1.0). Additionally, a clade containing both unicellular and filamentous taxa, including *Zygnemopsis minuta, Mesotaenium kramstai* and two strains of *Cylindrocystis*, was found (100/100/1.0). This clade appears to be equivalent to the MZC clade as described by Gontcharov et al. (2004).

Netrium digitus, classically part of the Zygnematales, was found to be sister to taxa traditionally classified among the Desmidiales. Within the lineage most consistent with the concept of the Desmidiales, most families were monophyletic: Gonatozygaceae (100/100/1.0), Closteriaceae (100/100/1.0) and Desmidiaceae (85/90/1.0) (Figure 3.1). *Penium* formed two paraphyletic lineages sister to the Desmidiaceae; one containing the taxa *P. cylindricum* and *P.* cf. *didymocarpum*, and the other *P. margaritaceum* and *P. spirostriolatum*. Monophyly of the Peniaceae was tested explicitly and found to be inconsistent with the present data based on an AU test ($p = 3e^{-3}$). Within the Gonatozygaceae, *Genicularia spirotaenia* was always embedded among species of *Gonatozygon*, usually sister to *G. pilosum* (100/99/1.0) (Figure 3.1). Placement of *Roya* spp., which may or may not be part of the Gonatozygaceae (Gontcharov et al. 2004), remains unclear but are supported as members of a lineage including most Desmidiales (95/100/1.0). When included in likelihood analyses of all nucleotide positions, *Roya* spp. appeared sister to a clade of *Gonatozygon* spp. (-/-/0.91).

In every analysis, *Phymatodocis nordstedtiana* Wolle was sister to all other Desmidiaceae (Figure 3.2). When using all characters, *Actinotaenium* spp. formed a monophyletic clade diverging after *P. nordstedtiana* but before all other Desmidiaceae (Figure 3.2). These remaining Desmidiaceae comprised two large monophyletic groups: one contained the filamentous and colonial genera (except *Phymatodocis*), and the other contained all unicellular taxa (except *Actinotaenium* spp.). This topology contrasts with some other published phylogenies (see discussion). Among the filamentous desmids, two lineages were resolved, one containing *Spondylosium tetragonum*, *Teilingia granulata*, and *Cosmocladium saxonicum* (100/100/1.0), and the other containing *Spondylosium pulchellum*, *S. pulchrum, Desmidium* spp, *Bambusina borreri, Hyalotheca* spp., *Heimansia pusillum* and *Onychonema leave* var. *micracanthum* (99/100/1.0) (Figure 3.2).

Among unicellular Desmidiaceae, *Haplotaenium minutum* (previously *Pleurotaenium minutum*) did not group with *Pleurotaenium* spp., supporting the taxonomic separation of these two taxa based on ultrastructural characteristics (Figure 3.2). *Triploceras gracile* was found embedded among species of *Micrasterias* with high support (99/100/1.0). This result was unexpected because these two taxa are structurally dissimilar.

Although most analytical methods resulted in similar topologies, some inferences were specific to a particular dataset, analytical method or model parameters. ML analysis resolved *Haplotaenium minutum* sister to other unicellular taxa, but the MP analysis found this species embedded among *Staurastrum* spp. with moderate (BS = 78) support. The MP tree also showed *Mesotaenium endlicherianum* and *M.* sp. JH0031 sister to *Netrium*.

While testing monophyly of genera was not the central intent of this study, the data refute the monophyly of some genera. In particular, *Cylindrocystis* spp., and *Mesotaenium* spp. occurred in two different clades, as did *Cosmarium* spp., *Euastrum* spp. *Spondylosium* spp. and *Staurodesmus* spp.. These results appear to be robust because they were found regardless of analytical method or dataset.

Individual gene analyses

Before concatenation, individual genes were analyzed separately to determine their utility for phylogenetic investigation and their combinability. Because of the number of taxa included, the short alignments from individual genes were generally poorly resolved and the deeper nodes received little statistical support. Some differences among the datasets were discovered.

The gene *rbcL* (base frequency A=0.28486 C=0.18748 G=0.22378 T=0.30388; length=1353 bp) had 656 variable sites, of which 568 were parsimony informative. The topology of the most likely tree was similar to that of the combined analysis. The major difference was that *Mesotaenium enlicherianum* was found outgroup to all other conjugating green algae, but without support for the placement (-/-/0.65), and *Penium* was present in three paraphyletic lineages, as opposed to the two found in the analysis of the concatenated dataset (data not shown). In the *rbcL* analysis, *Roya* was found sister to *Gonatozygon*, although this relationship was weakly supported (-/-/0.80).

The gene *psaA* (base frequency A=0.318557 C=0.124769 G=0.084047 T=0.472627; length=2118bp) was nearly twice as long as the *rbcL* sequence fragment and included two indels that were excluded from analyses. Of the 2073 included characters, 1052 were variable and 947 were parsimony informative. The topology of the most likely tree was very similar to that of the combined analysis, the difference being that *Penium* was placed into three rather than two paraphyletic lineages with low statistical support (64/66/0.94 for the sister relationship between *Penium cylindrus* and the Desmidiaceae). Additionally, *Roya* spp were found sister to the clade containing Closteriaceae, Peniaceae and Desmidiaceae (69/72/-).

The mitochondrial gene *cox*III (base frequency A=0.309707 C=0.131077 G=0.126975 T=0.432241; length=615bp) was the shortest gene included and had a single indel region, which was excluded from analyses. Of the 609 included nucleotides, 370 were variable and 334 were parsimony informative. The most likely tree estimated from this dataset was unlike that of the other two genes in that the deeper nodes were entirely unresolved including the separation from the outgroup taxa *Coleochaete* and *Chaetosphaeridium*. However, the midlevel relations were resolved with generally high bootstrap support (data not shown). Some species relationships, particularly among the Desmidiaceae, could not be distinguished based on their *cox*III sequence. Although the deeper nodes were unresolved, the relationships that were resolved were consistent with the topologies found in the *psaA*, *rbcL* and combined analyses.

Permutations of dataset

When third codon positions were removed from the combined dataset, support at deeper nodes declined, and relationships among genera of the Desmidiaceae were almost entirely unresolved. In ML analyses *Spirogyra* and *Sirogonium* were part of a monophyletic clade including most zygnematalean taxa that excluded *Netrium* and *Mesotaenium endlicherianum*. *Mesotaenium endlicherianum* was sister to all other conjugating green algae, and did not group with other *Mesotaenium* spp.. One noteworthy result of the exclusion of third position was that the filamentous lineage of Desmidiaceae, as previously described, was dismantled: the lineage containing *Teilingia* and *Spondylosium tetragonum* was sister to *Actinotaenium,* albeit with little statistical support (-/-/1.0).

Topology tests

Many of the relationships found are inconsistent with traditional classification. To test specific hypotheses of monophyly – particularly those posed by traditional classifications – constraint analyses were performed under the Likelihood criterion. The constrained nodes are listed in Table 3.3. These trees and the site-wise likelihoods were used to test if trees consistent with particular phylogenetic hypotheses were significantly worse than the best tree using the AU test. Three of the tested topologies were rejected by the data based on the AU test: monophyletic *Penium* (and Peniaceae) (AU= $3e^{-4}$), monophyletic *Mougeotia* and *Mesotaenium* (AU= 0.002), and monophyletic *Cylindrocystis* (AU= $3e^{-8}$) (Table 3.3).

Table 3.3 Constraints used in AU test

Constraint	AU	KH	SH
Monophyletic *Cylindrocystis*	**0.00000003**	**0.000**	**0.000**
Monophyletic *Gonatozygon* and *Roya*	0.496	0.402	0.959
Monophyletic *Mougeotia* and *Mesotaenium* excluding *M. kramstai*	0.089	0.088	0.285
Monophyletic *Mougeotia* and *Mesotaenium* including *M. kramstai*	**0.002**	**0.001**	**0.005**
Monophyletic *Penium*	**3E-04**	**0.002**	0.139
Monophyletic *Spirogyra*	0.592	0.482	0.953
Monophyletic *Zygnema*	0.617	0.518	0.967
Monophyletic Zygnematales incuding *Netrium*	0.404	0.391	0.831
Monophyletic Zygnematales w/o *Netrium*	0.330	0.299	0.939
Monophyletic Zygnematales without *Netrium* or *Spirogyra*	0.517	0.376	0.970

Discussion

Taxa

This study aimed to include all available genera of conjugating green algae and to broadly sample the structural diversity within these genera. Because of the great morphological diversity within the conjugating green algae, this is a challenging task. It was also complicated by some of the challenges inherent in maintaining large culture collections. Five strains from culture collections were contaminated, and four others were assigned names inconsistent with the species description. In most cases misidentified taxa had been assigned to the wrong genus. Consequently it was necessary to carefully identify the organisms prior to DNA extraction. This should not be interpreted as a criticism of culture collections, which often have to struggle with the complexities of maintaining a bewildering range of organisms on a minimal budget, but is a caution to all who make use of such culture collections. In this case, using the original strain designation would have strongly affected my interpretation of the relationships among the genera and caused us to erroneous conclude that many genera were not monophyletic.

There are several possible explanations for these misidentified strains, each of which carries its own implications. It may be that cultures were inadvertently mixed or contaminated by other strains in the collection during maintenance. It is also possible that the strains were misidentified at the time of submission. Without access to voucher material from the original isolates of many of these strains, it not feasible to determine the source of error with certainty. This emphasizes the importance of vouchering and identifying material at the time of analysis, even if it is available in culture collections.

Although efforts were made to identify every strain, it was not possible to do so with several of the filamentous Zygnematales. Taxonomy among these taxa is based on gametangial and zygospore characteristics. Most of these strains could not be induced to go through a sexual cycle. In particular, *Zygogonium tunetanum* was found to differ very little in primary sequence from *Zygnema circumcarinatum* in all genes sampled. The identity of neither strain could be verified, and the identification used here follows that of the culture collection. *Zygnema* and *Zygogonium* have been considered synonymous (Czurda 1932), but some authors separate the genera based on the shape of the chloroplasts (Transeau 1951). Both strains in this study have chloroplasts consistent with the genus *Zygnema*. More strains of *Zygogonium*, preferably freshly isolated and matched with vouchered field material, will be needed to establish the relationship between these genera.

No strains consistent with *Sphaerozosma* or *Groenbladia* were included in this study. Although several strains in culture collections are listed as *Sphaerozosma*, these strains are more consistent with *Teilingia*: the cells are relatively rectangular, lack apical processes and often have granules at the angles of the hemicells. *Spondylosium tetragonum* [*Groenbladia undulata*] SVCK 440 can be easily distinguished from *Groenbladia* both by the shape of the cell (which is more constricted than in *G. undulata*) and by the shape of the chloroplast. *Groenbladia* spp. have one or two axile taeniform chloroplasts while strain SVCK 440 has a lobed chloroplast similar to other species of *Spondylosium*. Additionally, *Staurastrum tetracerum* [*Arthrodesmus* sp.] UTCC 348 cells have small processes at the angles that are inconsistent with *Arthrodesmus* (now *Octacanthium* or *Staurodesmus*).

Multigene phylogeny

Because individual gene phylogenies were not incongruent with one another, the three genes were concatenated into a single dataset of over 4000 bp. This is a large dataset and two of the three markers have not previously been used for this lineage (*cox*III and *psaA*) although they have been used to estimate relationships in other algal groups. Single gene phylogenies, while topologically similar, had poor bootstrap proportions and low Bayesian posterior probabilities for several key branches. By combining these datasets, I was able to increase support for the resulting topology.

Combining the datasets, while improving support across most of the tree compared to individual gene analyses, did not result in full support for all nodes in the phylogeny. In particular, the deepest branches were not highly supported. In several cases, relationships with high posterior probabilities were not supported in bootstrap analysis. This may be because posterior probabilities tend to be somewhat of an overestimate for support (Douady et al., 2003; Simmons et al., 2004; Lewis et al., 2005).

In the *cox*III dataset, members of the Gonatozygaceae and *Roya* spp. were found to be somewhat divergent, however their positions were not strongly supported in bootstrap analyses (data not shown). Organisms classified in the Zygnematales, such as *Roya*, are characterized by smooth cell walls with an indehiscent primary wall. This character seems to be pleisiomorphic, and is found in several distinct lineages. Historically, the filamentous (Zynemataceae) and unicellular (Mesotaeniaceae) species with smooth walls were classified separately. Members of these two families are polyphyletic (McCourt et al. 1995, Gontcharov et al. 2003), and unicellular and filamentous organisms interdigitate in the phylogeny (Figure 3.1). These data suggest a number of transitions in growth form among these organisms. Assuming that the molecular phylogeny is correct, new morphological characters will need to found to permit morphological classification within the Zygnematales.

Chloroplast shape is one possibility (McCourt et al. 1995). Several lineages have similar chloroplast shapes: *Mesotaenium* and *Mougeotia*; *Zygnema* and *Zygogonium*; *Cylindrocystis* and *Zygnemopsis;* and *Spirogyra* and *Sirogonium*. The consistent placement of *Spirogyra* and *Sirogonium* as the basal-most lineage of the conjugating green algae is important and implies a unique status for these organisms. Unlike most conjugating green algae, which with a few exceptions have chloroplasts based in the center of the cell, *Spirogyra* and *Sirogonium* have parietal chloroplasts.

A second lineage where chloroplast shape appears to be informative is in the Desmidiales, including *Netrium*. In this clade, all the basal lineages are characterized by ridged central chloroplasts. Exceptions include apparently derived characteristics, such as the reduced lobes of *Gonatozygon* and the highly lobed and dissected chloroplasts of some Desmidiaceae. It also true that some species of *Spirotaenia* have a similar chloroplast, but placement of these taxa in the conjugating green algae is uncertain (Gontcharov & Melkonian 2004).

The placement of *Spirogyra* and *Sirogonium* as one of the earliest diverging lineages of conjugating green algae was observed in 18S rDNA analyses, but in that study their position was unresolved (Gontcharov et al. 2003). *Spirogyra* is not monophyletic with respect to *Sirogonium*, which differs from one previous study (Drummond et al. 2005), but is consistent with others (Gontcharov et al. 2003, Kim et al.

2006). The monophyly of *Spirogyra*, with the caveat that *Spirogyra maxima* is a *Sirogonium*, could not be rejected by these data based on the AU test (p = 0.592). It is not clear what is responsible for the different phylogenies discovered in different studies, but taxon sampling for *Spirogyra* and *Sirogonium* did vary widely among the studies.

The remaining Zygnematales (except *Netrium* spp. and *Roya* spp.) form a monophyletic clade with varying support (-/73/1.0). Two single-genus lineages are consistently found, one containing *Mesotaenium endlicherianum* and *M*. sp. JH0031 and the other containing *Cylindrocystis crassa* Bary and *C. brebissonii* Meneghini, although species of both *Mesotaenium* and *Cylindrocystis* occur in other clades as well (Figure 3.1). The monophyly of *Cylindrocystis* could be rejected by my data based on the results of an AU test ($p = 3e^{-8}$). A grouping of *Mougeotia* and all *Mesotaenium* strains was also rejected (p = 0.002). Besides the *Spirogyra* clade, three strongly supported zygnematalean clades were resolved. The first contains *Zygnema* spp and *Zygogonium tunetanum* (100/100/1.0). The second lineage includes *Mougeotia* spp. and *Mesotaenium caldariorum* (97/100/1.0). A relationship between *Mougeotia* and *Mesotaenium* would be predicted based on their structural and cytological characteristics: both have axile taeniform chloroplasts and both have similar cell wall structure (Brook 1982). The third lineage contains a mix of taxa including the filamentous *Zygnemopsis minuta* and the unicellular *Cylindrocystis* sp. UTEX, *C*. sp. JH0038 and *Mesotaenium kramstai* (100/100/1.0). Similar relationships were resolved using 18S rDNA and *rbc*L sequences (Gontcharov et al. 2004). Although these seem unlikely at first, it may be that there are characteristics that unite these taxa that have been overlooked.

Additionally, the Zygnematales are not monophyletic in analyses that use all codon positions, but are monophyletic (with the exclusion of *Roya* spp., *Netrium* spp., and *Mesotaenium endlicherianum*) when the third codon-positions are removed, but with little support (-/51/0.96). Monophyly of the Zygnematales could not be rejected by these data based on the AU test ($p = 0.404$) regardless of the exclusion of *Netrium* and *Roya* spp. ($p = 0.330$) or *Netrium*, *Roya* and *Spirogyra* ($p = 0.517$).

The Zygnematales clearly have a complex evolutionary history in their own right, but the majority of described species of conjugating green algae belongs to the Desmidiales. This latter order is monophyletic with high support (95/100/1.0) (Figure 3.1). The outgroup to the Desmidiales and distinct from other Zygnematales, albeit with low BS support (-/-/0.99), is a lineage containing *Netrium digitus* and *N. digitus* var. *lamellosum*.

Within the Desmidiales, the branching order of the families is generally well supported, although the placement of *Roya* is uncertain, and the families are monophyletic, with the exception of the Peniaceae. In this clade, the first lineage to branch includes species of *Gonatozygon* and *Genicularia spirotaenia* SAG 54.86. *Genicularia spirotaenia* is sister to *Gonatozygon pilosum* (100/99/1.0). Previous studies have suggested that this relationship necessitates the abandonment of the genus *Genicularia* (Gontcharov et al. 2003). The genus *Genicularia* is characterized by parietal spiraling chloroplasts (Bary 1848). However, this strain has what appears to be an axile chloroplast that is either complete or reticulate (Hall unpublished data), a condition not previously reported for the Gonatozygaceae. It is not clear if this strain is typical of *G. spirotaenia* or is an atypical strain of *Gonatozygon*; twisted axile chloroplasts has been reported for several species of *Gonatozygon* (Bary 1848), and further sampling of

Gonatozygon will be needed to clarify the situation.

The Peniaceae, comprising solely the genus *Penium*, is the only desmidialean family that appears to be non-monophyletic (Gontcharov et al. 2004; this study). In this study, these taxa form two paraphyletic clades. The first contains *Penium margaritaceum* and *P. spirostriolatum*; the second *P. cylindrus* and *P.* cf. *didymocarpum*. Both *P. margaritaceum* and *P. spirostriolatum* are elongate cells with pseudo-girdle bands (reminiscent of the Closteriaceae) and have thickened outer walls with linear ornamentation. The other lineage contains two species with less elongate cells and less conspicuous ornamentation. These results are consistent with those found by Gontcharov et al. (2004). The diagnosis of *Penium* spp. here is tentative, as I did not perform the TEM needed for unequivocal identification. These taxa warrant further morphological study and denser taxon sampling.

The Desmidiaceae were relatively poorly resolved, but several well-supported clades were consistently found. These include early-branching lineages of *Phymatodocis nordstedtiana* (85/90/1.0) and *Actinotaenium* spp. (89/90/1.0), a clade of filamentous or colonial species, and a clade of primarily unicellular species.

Actinotaenium spp. are found among the basal-most clades in the Desmidiaceae. One species, *Actinotaenium silvae-nigrae,* was recently moved from *Penium* based on its cell wall structure (Kouwets and Coesel 1984), a decision supported by the present data. Most unicellular species appear as a single lineage and most filamentous taxa in a second lineage. In the former, some *Euastrum* species and *Tetmemorus* form a monophyletic group (97/99/1.0). These genera share a characteristic apical incision, but *Euastrum* spp. are generally somewhat more compressed. *E. verrucosum* is not part of this clade and is found deeply embedded in a clade of *Cosmarium* spp.. *Euastrum verrucosum*, unlike other species of *Euastrum*, has a very shallow apical incision and its general shape has made its placement in any particular genus uncertain, although most authors have assigned it to *Euastrum*. The placement of this species among *Cosmarium* spp. suggests that many characteristics used to assign organisms to particular genera (such as the presence of lobes or an apical incision) may be homoplastic. For the moment, it would seem most appropriate to refer this taxon to *Cosmarium verrucosum* (Ehrenberg) Meneghini.

The position of *Triploceras gracile* among *Micrasterias* species is surprising. This relationship was moderately strongly supported in all analyses (99/100/1.0). *Micrasterias* species are much compressed and have highly dissected lateral lobes, while *Triploceras gracile* is elongate and radially symmetric at the isthmus. Explanations for this would include derivation of the genus *Triploceras* from a *Micrasterias*-like ancestor, more complex evolutionary histories such as organellar capture, lineage sorting or hybridization, and analytical artifact (although there is no particular reason to suspect such an artifact).

The *Staurastrum* species included in this study were found to be monophyletic. The species included span much of the known structural diversity of *Staurastrum,* from the spiny *S. polytrichum* f. *biseratum* to the long-processed *S. arctiscon*. This lineage does not include the monospinous species previously assigned to *Staurastrum* that could be assigned to *Staurodesmus.* Teiling (1948, 1965) assigned all monospinous species (those with a single spine at each angle of the hemicell), regardless of shape, to the genus *Staurodesmus*, including organisms with only mucrae. This study suggests that previous taxonomic treatments do not accurately reflect the evolutionary history of these subtly diverse organisms.

This analysis suggests that a number of desmid genera may not be monophyletic. *Cosmarium*, *Staurodesmus, Spondylosium* and *Euastrum* are found to be polyphyletic as suggested by previous studies (Gontcharov et al 2003, 2004; Gontcharov & Melkonian 2005). This study included only strains that could be identified with confidence. This excludes many published sequences because of the lack of adequate voucher information. If all related sequences available in GenBank were included, many additional genera would be non-monophyletic, but the significance of this observation is unclear.

In this analysis, most filamentous and colonial forms are found in a single clade with moderate support (83/84/0.97). Within this clade, there are two clear lineages, one containing *Cosmocladium saxonicum*, *Spondylosium tetragonum* and *Teilingia granulata* (100/100/1.0), and the other containing species of *Spondylosium*, *Desmidium*, *Hyalotheca*, *Bambusina*, *Onychonema* and *Heimansia* (99/100/1.0). These results support the separation of *Heimansia* from *Cosmocladium* (Coesel 1993) and suggest that the genus *Spondylosium,* as currently circumscribed, is untenable. Species of *Spondylosium* are resolved as three separate lineages. *Spondylosium pulchrum* is found sister to *Desmidium* spp., while *S. pulchellum* is an independent lineage sister to one of the two main clades of filamentous Desmidiaceae. Three strains here identified as *S. tetragonum* are found sister to *Teilingia granulata*. Previous analyses (Gontcharov et al. 2003) also found the genus *Desmidium* to be polyphyletic; this analysis did not include the same taxa as that study, and the strains here included form a monophyletic group sister to *Spondylosium pulchrum* (Figure 3.2).

In sum, this study used DNA sequences from multiple organellar loci to estimate the phylogeny of the conjugating green algae. These analyses suggest that these algae have a complex evolutionary history and that some systematic characteristics may be pleisiomorphic such as smooth indehiscent walls. Other characteristics will have to be determined before we can really understand the evolution of this group or predict to which lineage many organisms (particularly among the "zygnematales") belong. I suggest that cytological characteristics such as chloroplast shape and location may be useful characteristics. Careful observation of the organisms is necessary not only for the purposes of discovering structural or cytological synapomorphies of the clades resolved and described, but also to be certain that the data going into the phylogenetic analysis are as accurate as possible.

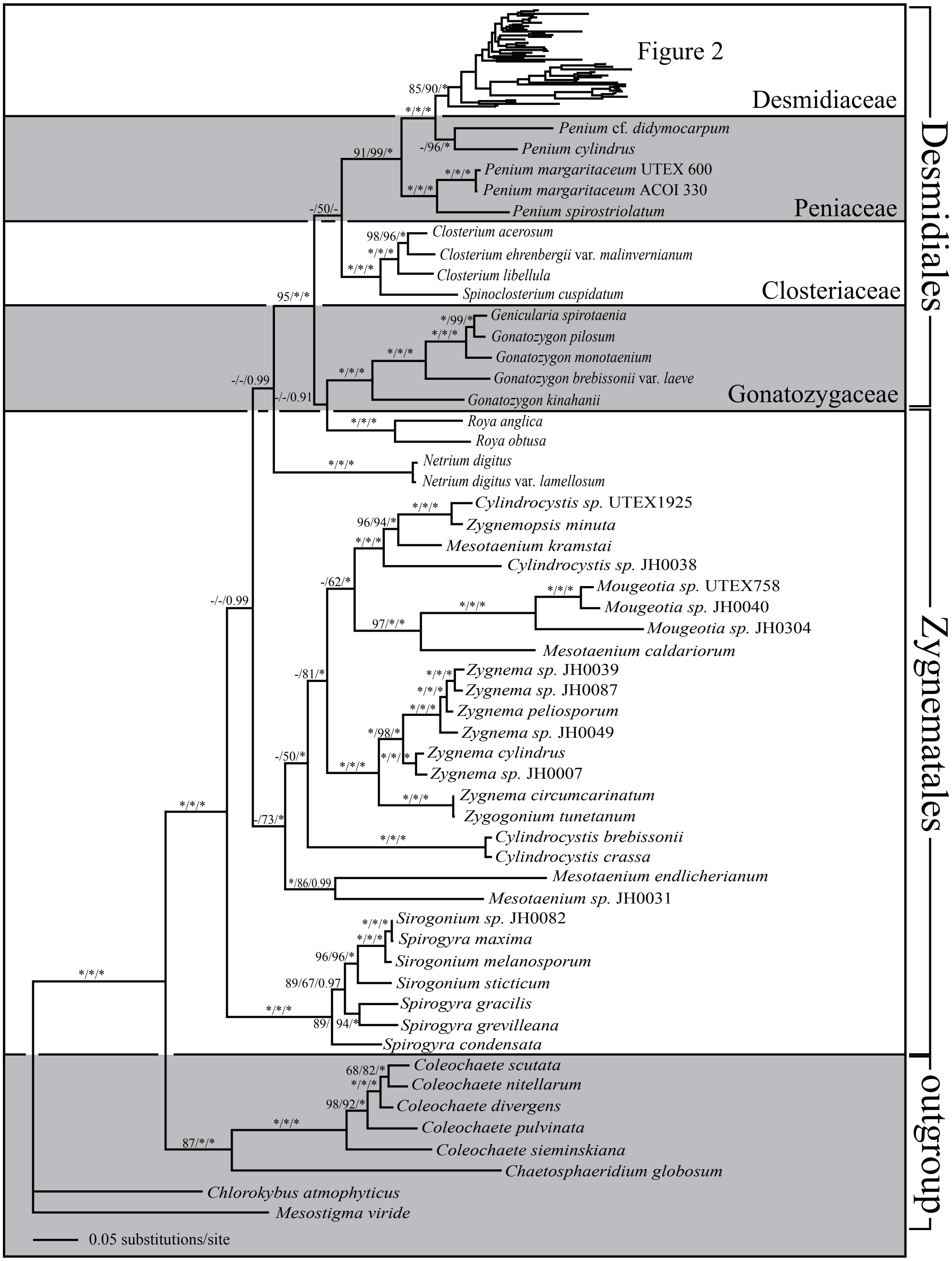


Figure 3.1. Phylogeny of the Zygnematophyceae based on *rbcL, psaA and cox*III. Tree based on the most likely tree when analyzing 3 genes with all codon positions included. Numbers above branches are ML and MP BS values and PP, respectively. Dashes indicate values less than 50% (BS) or .50 (PP). An asterisk indicates support values of 100 (BS) or 1.0 (PP).

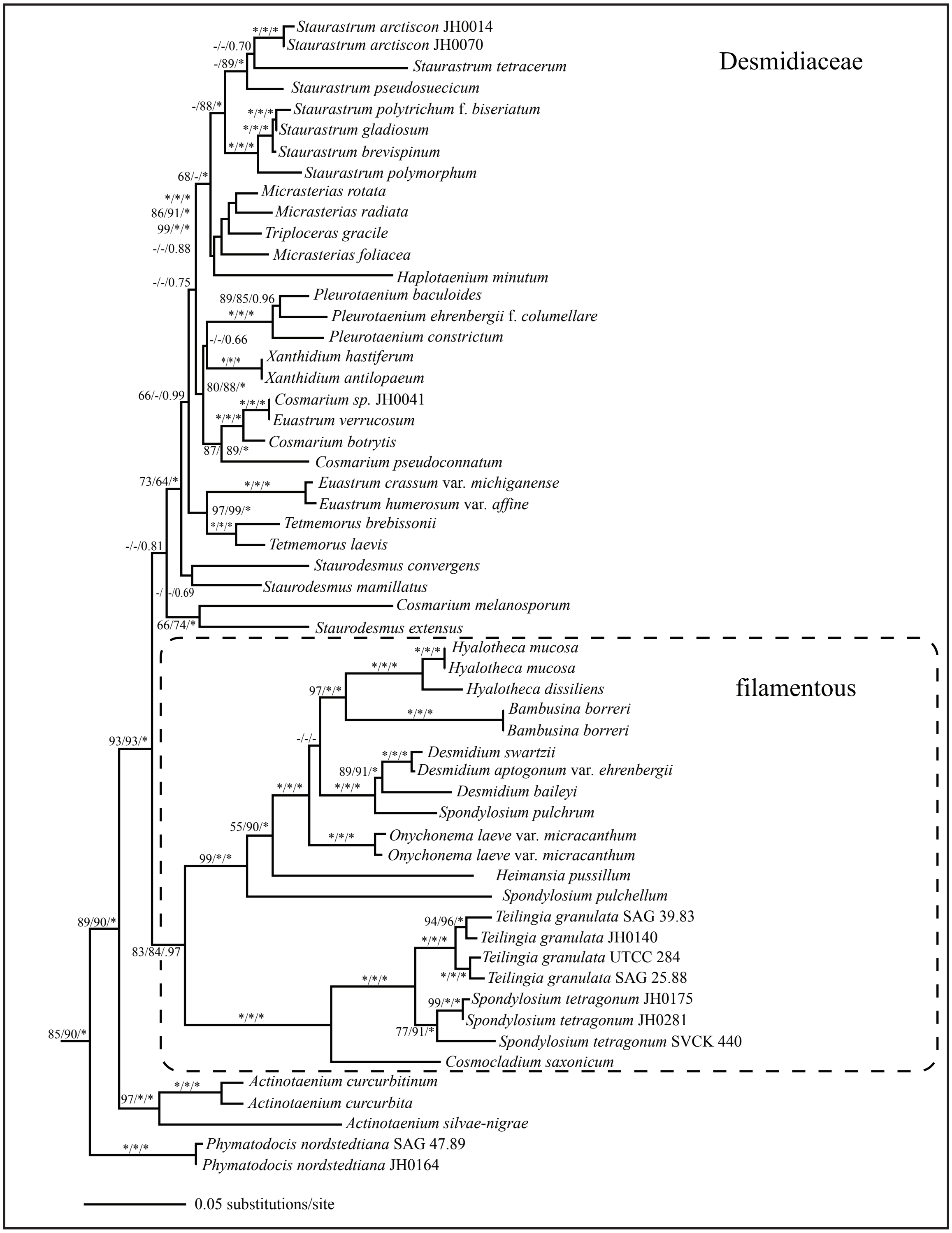


Figure 3.2. Phylogeny of Desmidiaceae based on *rbcL, psaA and cox*III. Enlarged portion of ML tree from figure 1 showing the phylogeny of the families of the Desmidiaceae based on the most likely tree when analyzing 3 genes with all codon positions included. Numbers above branches are ML and MP BS values and PP, respectively. Dashes indicate values less than 50% (BS) or .50 (PP). An asterisk indicates support values of 100 (BS) or 1.0 (PP).

Chapter 4. Patterns of cell division in the filamentous Desmidiaceae, close green algal relatives of land plants

Abstract

Patterns of cell division and cross wall formation vary among the charophyte green algae, the closest living relatives of land plants. The conjugating green algae are highly diverse and are known to display substantial variation in mode of cell division, but the details of these cell division patterns and their phylogenetic distribution remain poorly understood. I report here a study of cross wall development in filamentous Desmidiaceae (conjugating green algae) using light and fluorescence microscopy. All strains investigated showed centripetal encroachment of a septum, but with several distinctly different developmental patterns. In some cases cell wall formation is delayed with respect to the *Cosmarium*-type of cell division and the cross wall modified considerably after deposition in a manner specific to particular clades of filamentous desmids. These characteristics were mapped on a phylogeny estimated from a dataset of two organellar genes, and the evolutionary implications of the character state distribution were evaluated. The data suggest a complex history of evolution of cell division in this lineage, and also imply that *Desmidium* and *Spondylosium* are polyphyletic. These results suggest that cell division and the mature cross walls may be useful taxonomic characters for identifying filamentous conjugating green algae.

Introduction

Cell division is a fundamental process critical to the proliferation of all cells. In plants and many algae, once nuclei, organelles and other cytoplasmic contents separate, cells must undergo cytokinesis as well as the simultaneous construction of a new extracellular wall. Land plants share a single mode of cell division involving the use of a phragmoplast, but their close relatives, the charophyte green algae, show differences in their modes of cell division.

The nature of cell wall deposition plays an important role in determining the shape of the cell. In unicellular organisms this determines the structure of the organism, and changes in the process of cell division are considered important evolutionary steps in the evolution of complex cellular organization (Graham et al., 2000). In *Coleochaete*, for example, the ability to control the plane of cell wall deposition allows the filaments to bifurcate, and leads to the branched thallus characteristic of the genus. Transitions from unicellular to filamentous forms have occurred many times in the evolution of the conjugating green algae (Zygnematophyceae) (McCourt et al., 1995; Gontcharov et al., 2003). Their close relationship to land plants makes the lineage an exceptional model for studying the process of cell division and morphogenesis and provides direct insight into the evolution of multicellularity in the lineage that gave rise to land plants (Karol et al., 2001; Turmel, Otis, and Lemieux, 2006).

Many algae lack a cell wall, but all charophyte algae (except *Mesostigma,* which has scales), have a cellulosic cell wall that must form during division or subsequent development (Graham and Wilcox, 2000). Among charophytes, cell wall formation proceeds by centifugal growth of a cell plate (land plants, Charales and Coleochaetales) (Cook, Graham, and Lavin, 1998; Cook, 2004) or by centripetal encroachment of a peripheral septum (Zygnematophyceae, Klebsormidiophyceae, and *Chlorokybus*) (Graham et al., 2000). The conjugating green algae (Zygnematophyceae), however, are known to have several different variations of centripetal cell division (Brook, 1981). The diversity of patterns of cell division among the conjugating green algae is far greater than that found among land plants, but has received little investigation.

Many conjugating green algae undergo cell division as *Klebsormidium* and *Chlorokybus* do: via centripetal encroachment of a peripheral septum. Many constricted species -- that is, those that are divided into semicells connected by a narrow cytoplasmic isthmus -- exhibit the "*Cosmarium*-type" cell division in which the division septum forms soon after mitosis. The two new semicells then expand and the secondary wall is not deposited until the semicell is nearly fully formed (Pickett-Heaps, 1972; Meindl, 1993). In the Desmidiaceae, one of four families of conjugating green algae commonly referred to as desmids, the primary wall is then shed in its entirety or in fragments leaving only the secondary wall in mature cells.

Although the *Cosmarium*-type of cell division is common among constricted desmids it is not the only means of division for constricted cells. In *Onychonema laeve* Nordstedt, a filamentous desmid with constricted cells, a common vesicle forms between the semicells (termed a division vesicle). This vesicle enlarges to nearly the size of an adult semicell before a division septum forms (Krupp and Lang, 1985b). Once the wall forms, the semicells continue to grow and take the shape of their parent cell, in this case forming apical processes and lateral spines. The filamentous habit is maintained by a fragment of primary wall that is shared by adjacent cells, however, most of the primary wall is jettisoned as in other Desmidiaceae (Krupp and Lang, 1985a),

Perhaps the most remarkable variation of centripetal cell division is represented by *Bambusina borreri* (Ralfs) Cleve. In this alga, after the new cell wall forms (during cytokinesis), a cylinder of primary and secondary cell wall is deposited in the center of the cell. This results in what appears to be folds (replications) in the cross wall (see Figure 4.2K, L). As the cells elongate, these folds turn out and flatten, which results in a nearly full-size cell with a complete wall (Gerrath, 1973; Krupp and Lang, 1985b). This kind of cell division was thought to be typical of many filamentous desmid genera: *Bambusina*, *Desmidium*, *Streptonema* and *Haplozyga* (Krupp and Lang, 1985a).

Still other forms of cell division are known among the filamentous desmids. *Hyalotheca*, for example, shows simple centripetal cell division more characteristic of the Zygnematales than the Desmidiaceae (Acton, 1916; Krupp, 1980). Furthermore, cell division has not been explicitly studied in a number of filamentous genera and the mode of cell division is often inferred from the presence of cross walls of a particular shape, or cell division is assumed to be the same as that of superficially similar unicellular and filamentous species. Cell division patterns in all previously studied species are variations of centripetal cell division common to other charophyte and chlorophyte algae.

In the course of a molecular survey of the conjugating green algae and extensive field study, I observed undocumented variation in cell division among filamentous Desmidiaceae. To understand the phylogenetic and developmental significance of this variation, I undertook a detailed study of cell division in these taxa, and place that diversity in the context of molecular phylogenetic data. In addition to reporting characteristics of cell division for a number of genera and species, I also explored evidence concerning the evolution of centripetal cell division within the Zygnematophyceae as inferred from chloroplast and mitochondrial gene phylogenies.

Materials and Methods

Terminology

For the purpose of this paper, “cell division” refers to the entire process of division from pre-mitotic elongation of cells, through chloroplast division, mitosis, cytokinesis and the deposition of primary and secondary wall material. Chloroplast and nuclear division are referred to separately. A ”cross wall” refers not only to the primary wall or the position of wall deposition, but rather to the wall, sometimes with both primary and secondary material, that is deposited before a final stage of elongation in filamentous conjugating green algae. I follow previous authors in using terminology that parallels wall terminology for plants (Gerrath, 1973; Krupp and Lang, 1985b). The first wall deposited during cytokinesis is referred to as the primary wall and the subsequent thickened layer deposited inside the first wall as a secondary wall.

Culture conditions

Strains investigated were requested from public culture collections or isolated from the wild (Table 4.1). All strains were grown in Guillard’s Woods Hole medium (Nichols 1973) in a Percival growth chamber (Percival, Perry, IA) under Sylvania Cool White fluorescent lamps (Danvers, MA) and irradiance of 30 microeinsteins on a 16:8 hr light:dark cycle. at 15º C. Cells were observed at several times throughout the day with many cross walls visible several hours after the lights came on. Dividing cells could be found at any time of day. A list of strains used and accession information can be found in Table 4.1. Strains that were misidentified in culture collections are indicated in brackets. Synonymy is indicated with parentheses.

Table 4.1. Strains investigated and Genbank numbers

Taxon	Strain	*rbcL*	*cox*III
Bambusina borreri	JH0125	EF371284	EF371071
Closterium acerosum	UTEX 1075	EF371285	EF371072
Closterium ehrenbergii var. *malinvernianum*	JH0013	EF371286	EF371073
Closterium libellula	JH0021	EF371287	EF371074
Cosmocladium saxonicum	ACOI 95	EF371292	EF371079
Desmidium aptogonum	SVCK 108	**EF463091**	**EF463086**
Desmidium aptogonum var. *ehrenbergii*	JH0184	EF371298	EF371085
Desmidium baileyii	JH0228	EF371299	EF371086
Desmidium grevillei (*cylindricum*)	SVCK 113	**EF463090**	**EF463085**
Desmidium grevillii	JH0096	**EF463092**	**EF463088**
Desmidium swartzii	JH0042	EF371297	EF371084
Gonatozygon monotaenium	UTEX 1253	U71438	EF371092
Gonatozygon pilosum	ACOI 1096	EF371303	EF371093
Groenbladia taylorii	JH0339	**EF463093**	**EF463084**
Heimansia pusilla	SVCK	EF371291	EF371078
Hyalotheca dissiliens	SAG 384-2	AF203499	EF371095
Hyalotheca mucosa	JH0055	EF371305	EF371096
Micrasterias foliaceae	NIES 297	EF371311	EF371102
Micrasterias radiata	JH0064	EF371313	EF371104
Micrasterias rotata	UTEX 1941	EF371312	EF371103
Onychonema laeve var. *micracanthum*	JH0198	EF371318	EF371111
Onychonema moniliforme	JH0420	**EF463094**	**EF463089**
Penium margaritaceum	UTEX 600	EF371321	EF371114
Penium spirostriolatum	SVCK 205	EF371324	EF371117
Phymatodocis nordstedtiana	SAG 47.89	AJ553962_1	EF371118
Phymatodocis nordstedtiana	JH0164	EF371325	EF371119
Spondylosium pulchellum	SVCK 365	AF203505	EF371137
Spondylosium pulchellum	JH0368	**EF463096**	**EF463087**
Spondylosium pulchrum	JH0269	EF371341	EF371138
Spondylosium tetragonum	SVCK 440	AF203498	EF371094
Staurastrum arctiscon	JH0014	EF371343	EF371140
Staurastrum polytrichum f. *biseriatum*	JH0015	EF371344	EF371141
Teilingia granulata	SAG 39.83	EF371335	EF371130
Teilingia granulata	UTCC 284	AF203504	EF371131
Teilingia granulata [*Spondylosium planum*]	SVCK 418	**EF463095**	**EF463083**
Teilingia granulata [*Spondylosium secedens*]	SVCK 24	**EF463097**	----------

Notes:

ACOI, Coimbra Collection of Algae; NIES, National Institute for Environmental Studies; SAG, Sammlung von Algenkulturen der Universität Göttingen; SVCK, Sammlung von Conjugaten-Kulturen; UTEX, Culture Collection of Algae at University of Texas; UTCC, University of Toronto Culture Collection of Algae and Cyanobacteria. Vouchers of strains beginning with JH are available from the authors. Bold type sequences were generated specifically for this study.

Molecular phylogenetic analysis

DNA was extracted using the Nucleon Phytopure DNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ). The chloroplast gene *rbc*L and mitochondrial *cox*III were amplified by PCR using published primers (Chapter 3) and sequenced using Big Dye Terminator sequencing technology on an Applied Biosystems 3100 capillary sequencer (Applied Biosystems, Foster City, California). Fragments were sequenced in both directions and assembled using Sequencher 4.2 (GeneCodes, Ann Arbor, MI). Sequences were aligned in MacClade 4.7 (Maddison and Maddison, 2000). Phylogenetic analyses were performed in PAUP* (Swofford, 2003). Under the Parsimony criterion (MP), best trees were searched for heuristically with ten random taxon addition sequences and tree-bisection-reconnection (TBR) branch swapping. Support for taxon bipartitions was estimated using the non-parametric bootstrap (BS), with 500 pseudoreplicates and 10 random taxon addition sequences in each pseudoreplicate. Under the Maximum Likelihood criterion (ML) I used the GTR+I+G model of sequence evolution with parameters estimated from the data. One hundred bootstrap replicates were run with three random taxon addition sequences per replicate. Two analyses in MrBayes (BI), each using four chains sampled for four million generations, sampled every one hundred generations, were used to estimate the topology and posterior probabilities (PP) of the taxon bipartitions. The first two thousand trees were discarded as burnin.

Microscopy

Living cells were observed at various stages of cell division using a Zeiss Axioskop microscope (Zeiss, Germany). Image data were recorded as digital micrographs using an AxioCam HRc CCD camera (Zeiss, Germany). Many cells at different stages of cell division were observed, and when possible a single cell was followed through a division cycle. In most cases, cross walls were visible using differential interference contrast (DIC) microscopy, however, some stages were recorded by staining the cells with the cellulose-specific fluorochrome Calcofluor. When staining, live cells were first harvested by gentle centrifugation and fixed with 3% glutaraldehyde for 20 minutes. Cells were allowed to settle and rinsed once in Guillard's Wood's Hole medium, and then stained for one hour with 1% Calcofluor. Cells were destained by three ten minutes rinses in medium and viewed on the same microscope using an HBO 50 Mercury arc lamp (excitation near 395) and a long-pass emission filter of 470nm.

Results

Cross walls

Differences in timing of cellular events, such as cell wall deposition, among species were observed. Many differences in cross wall structure were only apparent in the fully mature cross wall, that is, the stage of development just before the cells elongated to form mature semicells. All cross walls formed by the centripetal encroachment of a peripheral septum. Complex features of the cross wall formed after cytokinesis and the deposition of primary wall material. Calcofluor effectively stained secondary wall material, however, the first walls deposited were not always visible when stained with Calcofluor, even if easily visualized with DIC. This was particularly noticeable in *Hyalotheca* spp., *Spondylosium pulchellum* Archer, *Teilingia granulata* (Roy & Bisset) Bourrelly and *Spondylosium tetragonum* West.

Teilingia granulata

Teilingia cells are somewhat rectangular and the semicells are connected across a narrow isthmus. In strains of *T. granulata*, following chloroplast division (or simultaneous with it), the cells elongated and formed a narrow connection about the width of the isthmus (Figure 4.2N). A cross wall then formed across this vesicle and the resulting semicells further elongated and inflated until the mirror image of the parent semicell was fully developed. Thus I observed a number of cells that were elongate without a cross wall (Figure 4.3 J3) and elongate cells with a separating wall (Figure 4.3 J4).

Spondylosium tetragonum

Cells of *S. tetragonum* are nearly rectangular and have a broad isthmus. Consequently, the elongate cells were less obvious than in *Teilingia granulata*. Early stages of cell division were not observed in this species, however a number of elongate cells were observed (Figure 4.3 K4). Cells elongated very little before depositing a cross wall. Later stages were consistent with division similar to that of *Teilingia granulata* (Figure 4.3 K5, K6). Because of the small size and variability in cell length, key characteristics of cell division were at the limit of the techniques used here and confident interpretation of events would require more study.

Spondylosium pulchellum

S. pulchellum forms filaments composed of cells that are markedly constricted, compressed, and have a trapezoidal shape. Both strains observed underwent cell division via the *Cosmarium*-type cell division (see Figure 4.3I). Semicells were observed to disassociate slightly at the isthmus and formed a cross wall at the isthmus very early in cell division (Figure 4.2M). The resulting semicells expanded as a papillum-like protrusion that inflated to form a large hemisphere, ultimately taking the shape of a trapezoidal hemicell. Portions of the primary wall dehisced.

Hyalotheca dissiliens and *H. mucosa*

Hyalotheca cells are cylindrical and very subtly constricted in the mid region. In both species cells elongated very little before a wall was deposited. Encroachment of a peripheral septum was observed. This cross wall was irregular initially, but became linear as it thickened. The daughter cells then further elongated and pores appeared.

Groenbladia taylorii

Cells of *G. taylorii* are cylindrical and not noticeably constricted. When *G. taylorii* elongated before cytokinesis, it was apparent in some Calcofluor stained cells that new wall material was deposited at the isthmus (Figure 4.2O). Encroachment of a peripheral septum was not observed but cross walls and subsequent elongation were consistent with the hypothesis that *G. taylorii* underwent simple centripetal cell division much like *Hyalotheca* spp. and probably very similarly to *Spondylosium tetragonum*.

Bambusina borreri

Bambusina cells are cylindrical, often inflated in the midregion and almost always longer than broad. In *B. borreri*, cells elongated slightly before cytokinesis. A plane cross wall was deposited at the median. A cylinder of primary and secondary wall material formed in the center of the cell (Figure 4.2K). This cylinder then unfolded and turned out as the cell elongated (Figure 4.2L). Only the center portion, which is the point of connection between adjacent cells, remained intact. See Gerrath (1973) for details.

Desmidium grevillei (Kütz.) Bary

Cells of *D. grevillei* are cylindrical, broader than long, and connected along the entire apex. Early in cell division, cells of *D. grevillei* elongated, sometimes resulting in what appeared to be a shared vesicle (Figure 4.3 E4). The cell first divided by a cross wall and then a cylinder of cell wall material deposited in the center (Figure 4.2E, F). These folds were about 2 or 3 µm in amplitude compared to the nearly 5 µm folds found in *B. borreri*.

Onychonema laeve var. *micracanthum* Nordst. and *O. filiforme* (Ehr.) Roy & Bissett

Semicells of *Onychonema* are reniform and connected by a narrow isthmus. Before cytokinesis, cells of *Onychonema* form a large shared vesicle (division vesicle) between the semicells (Krupp and Lang, 1985a; this study). A division septum then divided the vesicle somewhat asymmetrically (Figure 4.2I, J). Once separated, the new daughter semicells rounded out at the angles and continued to expand. After the wall was deposited, the apical processes characteristic of *Onychonema* formed. This is the first description of cell division in *Onychonema filiforme*.

Desmidium aptogonum Bréb.*,* *D. aptogonum var. ehrenbergii* Kütz.*,* *Desmidium baileyi* (Ralfs) Nordst.*,* and *D. swartzii* (Agardh) Agardh

Cells of these species are angular in apical view, broader than long and connected by apical processes leaving open space between adjacent cells. Cells first elongated forming a shared vesicle at the isthmus (Figure 4.2C, D, G, H and Figure 4.3, C3). This vesicle was then divided by a plane cross wall. Unlike *Bambusina*, a cylinder of cell wall material was deposited at each angle of the semicell at the location where the cells would be connected by apical processes rather than in the center of the cell (Figure 4.2 C, D, G, H). The number of such cylinders correlated to the number of apical processes, found in the cell: the biradiate *Desmidium aptogonum* var. *ehrenbergii* had two; the triradiate *D. baileyi* had three and quadriradiate forms of *Desmidium* had four (observed, but none included in this study). The size of these cylinders was approximately proportional to the length of the apical processes of the mature cells. *Desmidium baileyi*, which has long process (>5µm), had the largest fold (also >5µm). These cylinders were apparent when the cells were stained with Calcofluor but difficult to see in untreated cells. *Desmidium swartzii*, which has very short processes, also had very short replicate folds, each fold being between 1 and 2µm in length. This made visualization with Calcofluor fluorescence difficult. Using DIC, the folds were visible but obscured by cytoplasmic contents in living cells.

Spondylosium pulchrum (Bailey) Archer

Cells of *S. pulchrum* are very deeply constricted and have a small apical process that is the point of connection between adjacent cells. Cell division in *S. pulchrum* differed from that of other *Spondylosium* species investigated. In this species, the cells initially elongated and formed a division vesicle similar to that found in *Onychonema*. A cross wall then divided the vesicle and a small cylinder of cell wall material was deposited in the center of the cross wall. The replicate fold was very small and appeared, as in *Desmidium swartzii*, as a small bubble on the cross wall (Figure 4.2 B). This mode of cell division was most similar to that of *Desmidium baileyi*, differing primarily in the scale of the features.

Micrasterias foliacea Bailey*, Phymatodocis, Heimansia* and *Cosmocladium*

Cell division patterns of two filamentous and two colonial desmids included in the phylogeny were not investigated. These were *Phymatodocis*, *Micrasterias foliacea*, *Heimansia* and *Cosmocladium*. These are all thought to use the *Cosmarium*-type cell division (see *Spondylosium pulchellum* in this study). My observations were consistent with this hypothesis but insufficient to confirm it. Only division in *Micrasterias foliacea* has been studied in detail (Lorch and Engels, 1979) and was found to be of the *Cosmarium*-type.

Molecular phylogeny

Phylogenetic analyses were performed on a dataset containing fragments of the chloroplast gene *rbcL* and the mitochondrial gene *cox*III. Outgroup species were selected from Chapter 3 and species within the filamentous clade were added to this analysis. Many other strains of filamentous Desmidiaceae were screened for differences in cross walls.

With the exception of *Phymatodocis* and *Micrasterias foliacea*, a single lineage of filamentous and colonial species was resolved with moderate statistical support (Figure 4.1) (90, 98, 1.0; MP, ML and PP). Within this clade, two lineages were resolved: one containing *Teilingia granulata*, *Spondylosium tetragonum*, and *Cosmocladium saxonicum* Bary; the other containing the remaining filamentous or colonial species (Figure 4.1). In the first lineage, *Cosmocladium*, a colonial desmid connected by delicate cell wall strands, diverged first. *Spondylosium tetragonum* was sister to a clade of *Teilingia granulata*. *Teilingia* strains showed some sequence and structural diversity (see discussion).

The second lineage included the remaining filamentous and colonial forms. *Spondylosium pulchellum* was the first to branch and was represented by two strains that were structurally very similar. *Heimansia pusilla* (Hilse) Coesel separated from other strains with moderate support (83/85/1.0). A lineage containing *Desmidium aptogonum, D. swartzii, D. aptogonum* var. *ehrenbergii*, and *D. baileyi* as well as *Spondylosium pulchrum* was resolved with strong support (100/100/1.0). Placement of *Onychonema* spp. was less supported, but the two species included, *O. laeve* var. *micracanthum* and *O. fililiforme*, were strongly supported as monophyletic (100/100/1.0). Two species of *Hyalotheca* were also monophyletic with strong support (100/100/1.0). The strains of *Hyalotheca* were part of a lineage that included *Groenbladia taylorii* (but not *Spondylosium tetragonum* = "*Groenbladia undulata*"), as well as *Bambusina borreri, Desmidium grevillii* and *D. cylindricum*. *Desmidium* was, therefore, polyphyletic, and the cylindrical forms were sister to *Bambusina* while the angular forms (mostly triangular in apical view) were sister to *Spondylosium pulchrum*.

Discussion

Several modes of cell division are well characterized among the conjugating green algae (Brook, 1981). Of the two orders of conjugating green algae, the Zygnematales and the Desmidiales, this diversity is concentrated among the latter. The filamentous Zygnematales, use a simple mode of centripetal cell division involving the encroachment of a peripheral septum. In one species of *Spirogyra*, cell division also involves a cytoskeletal array similar to a phragmoplast (Fowke and Pickett-Heaps, 1969a; Fowke and Pickett-Heaps, 1969b). Among the Desmidiales, the most common form of cell division is the *Cosmarium*-type. The filamentous Desmidiales demonstrate still more diversity in their modes of cell division and these modes are compared to the *Cosmarium*-type for convenience.

Some filamentous Desmidiales use the *Cosmarium*-type cell division. *Spondylosium pulchellum, Cosmocladium saxonicum, Heimansia pusilla* and the distantly related *Micrasterias foliaceae* and *Phymatodocis nordstedtiana* all use the *Cosmarium*-type cell division. *Teilingia granulata* and *Spondylosium tetragonum* delay primary wall deposition until after and initial phase of elongation. This results in the formation of a vesicle in *Teilingia* and elongate cells in *Spondylosium tetragonum*. These “vesicles” do not inflate which distinguishes them from similar structures in *Onychonema* and *Spondylosium pulchrum*.

Among the remaining filamentous desmids, there are four unique modes of cell division. Cell division in *Groenbladia taylorii* and *Hyalotheca* is similar to that of *Spondylosium tetragonum*. In these species, cells elongate without forming an inflated division vesicle. In *Onychonema*, deposition of primary wall material is delayed until after an initial phase of elongation (Krupp & Lang, 1985b; Figure 4.2I). The vesicle that results inflates to nearly the size of a mature semicell before it is divide transversely by a wall. The apical processes begin to form soon after cytokinesis, but are not apparent on the mature cross wall.

In the previously discussed species, differences in their cell division are related to the timing and degree of cellular processes. All delay deposition of cell wall material with respect to the *Cosmarium*-type. The inflated vesicle of *Onychonema* is similar to the early developing semicells of *Cosmarium* in that the vesicle consists of mostly primary wall material (Krupp et Lang, 1985b).

The cylinders of cell wall material deposited on the mature cross walls of *Desmidium* and *Bambusina* share no known homologues among green algae. A similar structure is found in some *Spirogyra* spp., however, in that organism the cylinders are present on the mature cells and are not part of a transitory developmental phase as in *Desmidium* and *Bambusina*. Convergence of two lineages of desmids on this similar mechanism would be surprising and the apparent distribution may be the result of losses rather than convergence. Nevertheless, these lineages are separated, phylogenetically, by organisms with other modes of cell division. Additionally, the two lineages differ somewhat in the placement of the cylinders on the mature cross wall. In the case of *Bambusina borreri* and *Desmidium grevillei*, a single cylinder is found in the center of the cell. In *Spondylosium pulchrum* and *Desmidium baileyi*, as well as other species of *Desmidium*, the cylinders are positioned at the site of future apical processes and vary in number as a function of the number of apical processes.

These observations are supported by dozens of published images of cells in the process of division. A table of some of these images is provided (Table 4.2). Only two images appear to be inconsistent with my results. In one case the image may depict a cell that has almost completed cell division (Förster, 1974). In the other case (Förster, 1964), there is no obvious explanation for the discrepancy except to say that the diagnostic characteristics in that species would be very small and the previous report was based on fixed material and, most likely, a single or very few cells. Other images showing a flat division plate in *Desmidium* and *Bambusina* would seem to be inconsistent with my results, however, in these species a flat cross wall is initially deposited and the characteristic cylinders of cell wall material develop subsequently. Therefore these images are not in conflict with my results.

When first observed, the shared vesicle found in *Teilingia* was thought to be the product of a failure of cytokinesis. This is a common mutation in constricted desmids that gives rise to "giant" cells. These mutant cells are maintained through successive divisions, though it is possible for them to give rise to typical-shaped cells (Brook, 1981). In this case, cells were followed through a division cycle and found to separate normally.

Most filamentous Desmidiaceae were resolved in a single lineage in this analysis, however, another study based on 18S rDNA sequences found two clades of filamentous desmids (Gontcharov et al., 2003). These two clades are probably analogous to the clades described here (Figure 4.1). The two clades contain organisms with similar modes of cell division. However, even though cell division in *Teilingia granulata* is similar to cell division in both *Onychonema* and *Groenbladia taylorii,* this is very likely an independently derived state. These species were resolved in separate clades among organisms with other modes of cell division (Figure 4.4).

Basal nodes in the second lineage of filamentous desmids were not strongly supported (-/52/1.0) and the relationships between *Heimansia pusilla*, *Spondylosium pulchellum* and the lineage containing *Hyalotheca* and *Desmidium swartzii* were weakly supported (Figure 4.1). In this clade, *Onychonema* exhibits a unique mode of cell division (Figure 4.4). The presence of a replicate fold on the mature cross wall unites *Spondylosium pulchrum* with several angular species of *Desmidium*, which are monophyletic (100/100/1.0). *Desmidium grevillei* and *Bambusina borreri* formed a monophyletic clade with strong support (89/98/1.0) and shared a form of cell division involving the deposition of a single cylinder of wall material following cytokinesis (Figure 4.2 E, F, K, L). *Desmidium* is, therefore, polyphyletic. *Desmidium* spp. with an exactly similar cross wall shared a common ancestor with *Bambusina borreri,* while other species of *Desmidium* did not. These two groups of *Desmidium* are clearly distinct phylogenetically and structurally (Gontcharov et al. 2003; this study).

Two previous studies found that species of *Spondylosium* were polyphyletic (Gontcharov et al., 2003, 2004). Part of this confusion resulted from the misidentification of two strains of *Teilingia* (previously reported as *S. planum*, and *S. secedens*) (Chapter 3). A polyphyletic *Spondylosium* was also found in this study. I believe that the strains are now correctly identified and the apparent phylogenetic relationships of the organisms are supported by the observation that the three lineages exhibit three different kinds of cell division (Figure 4.4). The clade of *Teilingia granulata* shows more sequence diversity than one might expect. In addition to this sequence diversity there were also structural differences between the strains. Strains differed in cell sizes and the degree of constriction (data not shown). Within each strain, some cells lacked the apical granules characteristic of the genus.

Although several modes of cell division were known for conjugating green algae, the species investigated in this study are unique in that they are filamentous representatives of a species rich and mostly unicellular family, the Desmidiaceae. Accordingly, cell division in this lineage brings some evidence to bear on the evolution of multicellularity in charophyte green algae. There are few desmid lineages that contain filamentous species. *Phymatodocis* is one such organism and seems to represent an entirely independent evolution of the filamentous state. *Micrasterias foliacea* also forms filaments, however, in this species the filaments are held together by the interlocking of apical processes, not by means of a shared primary wall (Lorch and Engels, 1979). It is also true that this "filamentous" lineage contains organisms which are better described as colonial, namely *Cosmocladium saxonicum* and *Heimansia pusilla*. These species have still other means of connecting cells to one another (Gerrath, 1970). It is not clear why there are so few transitions from unicellular to colonial and filamentous forms among the desmids. In a related group, the Zygnematales, organisms have made the transition between unicellular and filamentous forms several times (McCourt et al., 1995; McCourt et al., 2000; Gontcharov, Marin, and Melkonian, 2003, 2004; Chapter 3).

These results provide some indication of the plasticity possible in the process of cell division. While the cells in these filaments most likely do not share cytoplasmic connections, the cells, at the very least cooperate with one another to form filaments of diverse architecture. The differences in cell division are the product of not only changes in the timing and order of cellular events, but also the evolution of novel structures. This demonstrates that variation is possible in processes as complex and critical as cell division.

It is clear that there is much diversity of cell division among the filamentous conjugating green algae, but the exact details of these differences remain obscure. I investigated cross walls as structural characteristics and to some degree their formation, but other aspects of cell division are important for understanding the evolution of these cross walls such as timing of chloroplast and nuclear division, and the fate of the primary wall. All of these require more in-depth study of each species and probably the employment of different methods such as TEM. This study is only the beginning of such an investigation. Future work may reveal more information that will be useful in determining the evolutionary history of cell division in this unique group of organisms.

Table 4.2. Published images of cell division in Desmidaceae

Taxon	Citation	Type	Consistent	Note
*Bambusina borreri**	Gerrath 1975	Bambusina	Yes	
*Bambusina borreri**	Hauptfleisch 1888	Bambusina	Yes	
*Bambusina borreri**	Krupp & Lang 1985b	Bambusina	Yes	
Desmidium aptogonum var. *acutius*	Förster 1964, pl. 36, fig. 2	Bambusina	No	
Desmidium baileyi	Förster 1974, pl. 36, fig. 5	Bambusina	No	late stage?
Desmidium baileyi	Gerrath 2003, fig. 50	Desmidium	Yes	
Desmidium baileyi	Hauptfleisch 1888	Desmidium	Yes	
Desmidium baileyi	Scott & Prescott 1961, pl. 62, fig. 8,10	Desmidium	Yes	
Desmidium baileyi	Scott et al. 1965, f. 247	Desmidium	Yes	
Desmidium baileyi	Smith 1920, pl. 88, fig. 7	Desmidium	Yes	
*Desmidium grevillei**	Couch & Rice 1948, fig. 11	Bambusina	Yes	
Desmidium grevillei	Smith 1920, pl. 88, fig.1	Bambusina	Yes	late stage
Desmidium grevillei	Tell & Domitrovic 1992, pl. 2, fig. 13b	Bambusina	Yes	late stage
*Desmidium swartzii**	Hauptfleisch 1888	Desmidium	Yes	
*Hyalotheca dissiliens**	Acton 1916	Hyalotheca	Yes	
*Hyalotheca mucosa**	Hauptfleisch 1888	Hyalotheca	Yes	
*Onychonema laeve**	Krupp & Lang 1985a	Onychonema	Yes	
Onychonema laeve var. *sumatrana*	Scott & Prescott 1961, pl. 61, fig. 1	Onychonema	Yes	
Spondylosium pulchellum	none			
Spondylosium pulchrum	none			
Spondylosium tetragonum	none			
Teilingia granulata	none			
Teilingia spinulosa	Palamar-Mordvinseva 2005, pl. 149, fig. 12	Teilingia	Yes	
Teilingia wallichii [as *Sphaerozosma wallichii*]	Turner 1892, pl. 18, fig. 1	Teilingia	Yes	

* These studies specifically mention the cross walls in the publication

Type – type of cell division consistent with published image

Consistent – whether or not the image is consistent with my findings

Note – comment on possible source of inconsistency

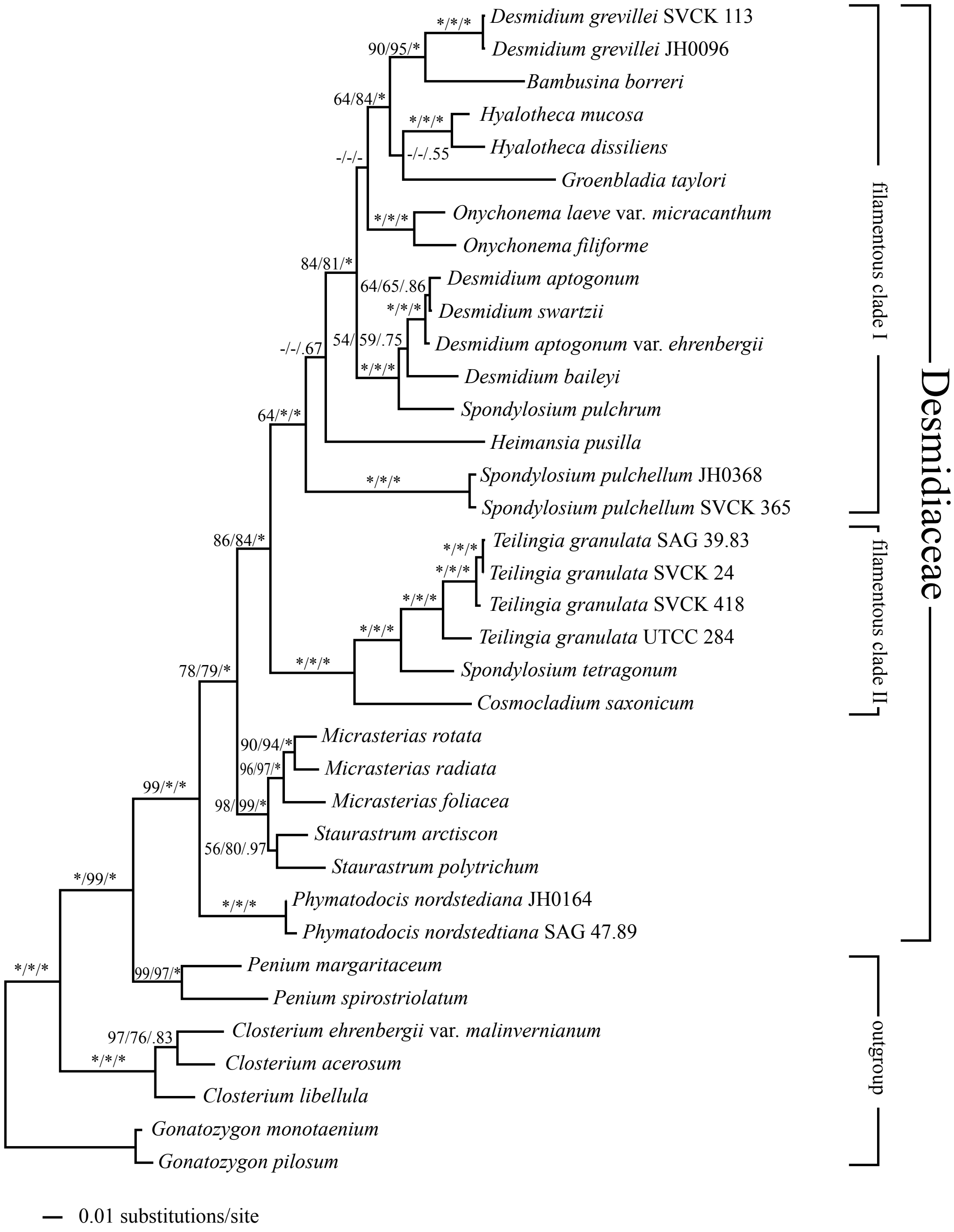
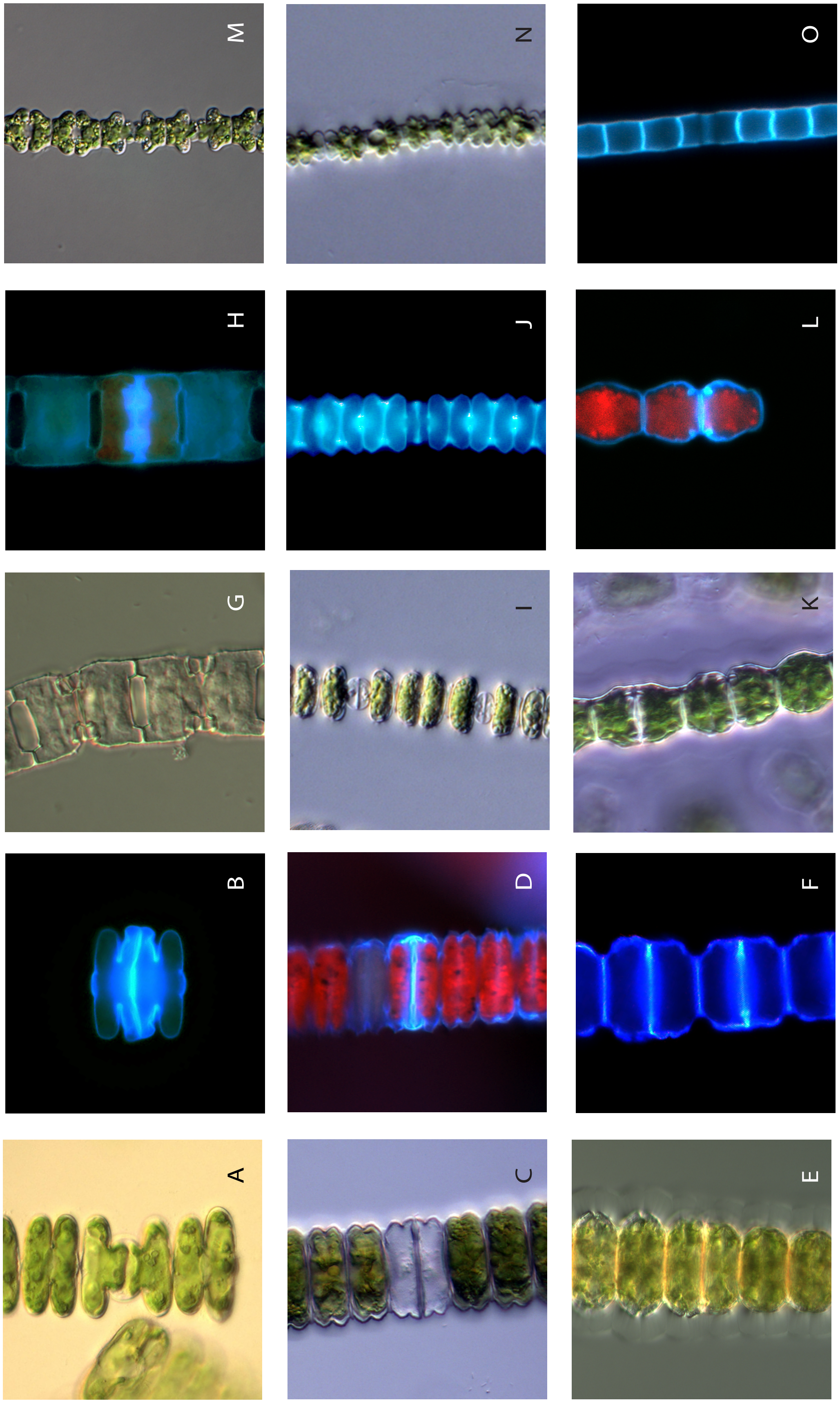


Figure 4.1. Phylogeny of the filamentous Desmidiaceae based on *rbcL* and *cox*III gene sequences. Topology based on the ML tree. Numbers above branches are bootstrap values from Parsimony and Maximum Likelihood and Posterior Probabilities from Bayesian Inference. Bootstrap values of 100 and posterior probabilities of 1.0 are indicated by an asterisk (*).

Figure 4.2. A-O. Various stages of division in filamentous Desmidiaceae. A. Light micrograph of early vesicle formation in *Spondylosium pulchrum*. Chloroplast has divided, but no wall material has been deposited. B. Fluorescence micrograph of Calcofluor stained *S. pulchrum* showing development of lobes and the presence of a small cylinder of cell wall material in the center of the cell. C. Light micrograph of *Desmidium swartzii* showing the small cylinders of cell wall material near the angles of the cell. D. Fluorescence micrograph of *D. swartzii* showing cylinder of material at angles of cell, new wall deposition. Red is autofluorescence from the chloroplast. E. Light micrograph of *D. grevillii* showing a late stage of cell division with folds at the angles, visible as highly refractive spots on the cell plate. F. Fluorescent micrograph of Calcofluor stained *D. grevillii*. Late stage showing mature cell plate. G. Light micrograph of *D. baileyi* fixed in the final stages of cell division. Edges of cell have pulled apart and cylinders of cell wall material are clearly visible at the angles. H. Fluorescence micrograph of an earlier stage in cell division of *D. baileyi*, before the edges of the cells have pulled apart. I. Light micrograph of early stage of cell division in *Onychonema filiforme*. Division vesicle is fully formed and the primary wall has been deposited across the vesicle. J. Fluorescence micrograph of early stage cell wall deposition in *O. filiforme*. K. Light micrograph of cell division in *Bambusina borreri* showing folds of cell wall material. Upper cell in a slightly earlier stage than the lower which has already pulled apart at the edges and is beginning to elongate. L. Fluorescence micrograph of *B. borreri* showing a late stage of cell division. Cylinder of cell wall material is fully formed and the cells have begun to move apart turning the folds inside out. M. Light micrograph of cell division in *Spondylosium pulchellum*. Septum forms very early in division and the daughter hemicells form from the isthmus of their parent hemicell. Two cells are in this stage of division. N. Light micrograph of cell division in *Teilingia granulata*. Vegetative stage cells visible at the bottom of the micrograph. Cells elongate at the isthmus forming a rectangular vesicle that is later divided by a septum. Three cells in the center of the micrograph have formed walls across this vesicle. O. Fluorescence micrograph of *Groenbladia* taylorii showing, regions of new wall deposition along edges of cell as well as the position of the primary wall across the dividing cell.


A
B
C
D
E
F
G
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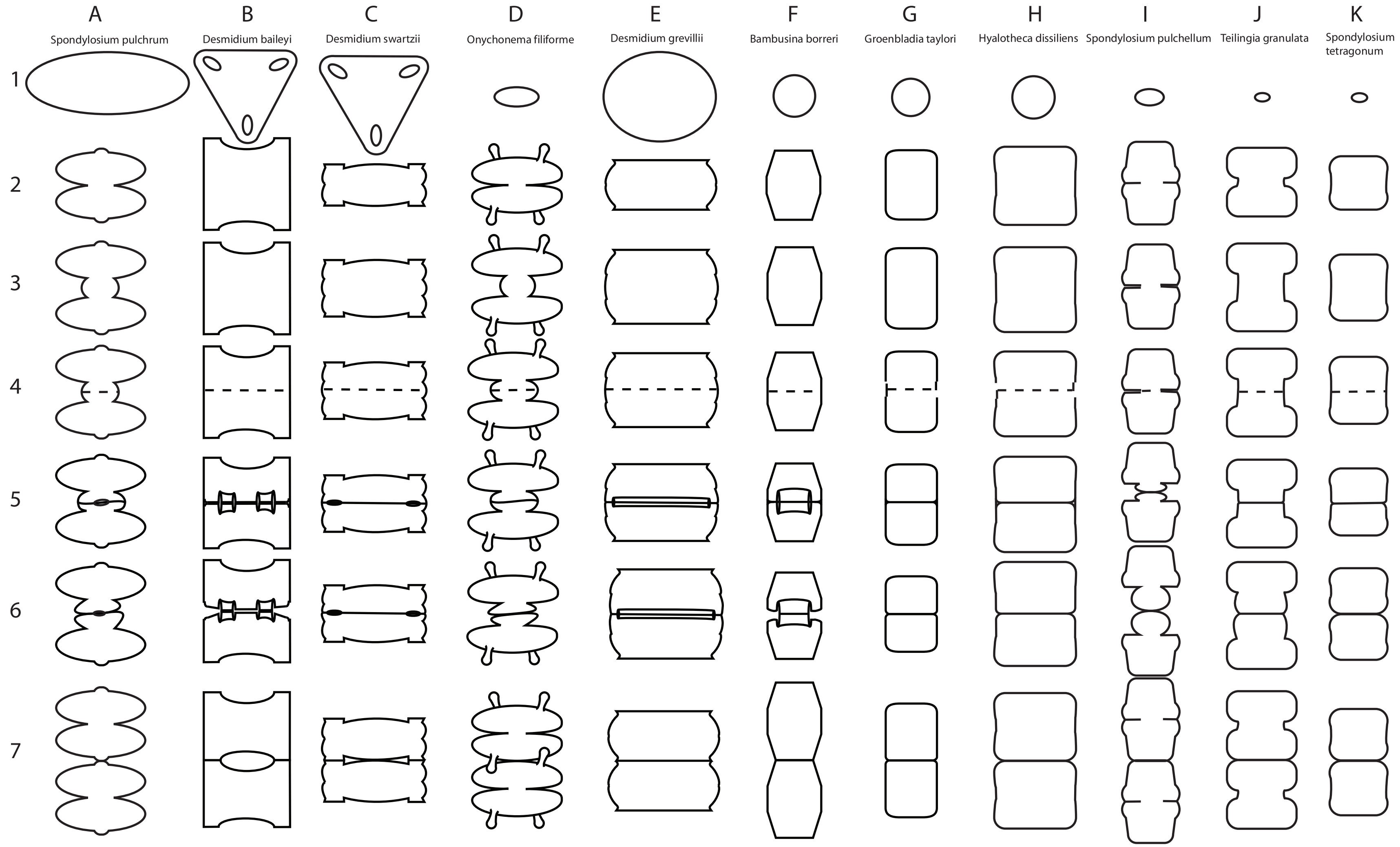
O

Figure 4.3. Model of cell division in eleven different species of filamentous Desmidiaceae. Row 1 shows the cells in apical view and is scaled to show the relative size of the organisms. *Spondylosium tetragonum* is, on average, about 5 µm wide and *S. pulchrum* nearly 60 µm wide. Other rows are scaled arbitrarily, but consistently within the column, to show the features of cell division plates. Dashed lines show the plane of primary wall deposition, and where new wall has clearly been deposited in *Groenbladia undulata* and *Hyalotheca dissiliens*. Besides these, no distinction is made in this figure between primary and secondary cell walls. Row 2 shows a normal vegetative cell; row 3 shows cells at the end of the predivision elongation; row 4 shows the plane of cell division and aspect of the cells at the time of primary cell wall deposition; row 5 shows the mature cell plates; row 6 shows the post cytokinesis elongation of the cells; row 7 shows the juxtaposition of the resulting cells in a filament.

A
Spondylosium pulchrum
B
Desmidium baileyi
C
Desmidium swartzii
D
Onychonema filiforme
E
Desmidium grevillii
F
Bambusina borreri
G
Groenbladia taylori
H
Hyalotheca dissiliens
I
Spondylosium pulchellum
J
Teilingia granulata
K
Spondylosium tetragonum
1
2
3
4
5
6
7

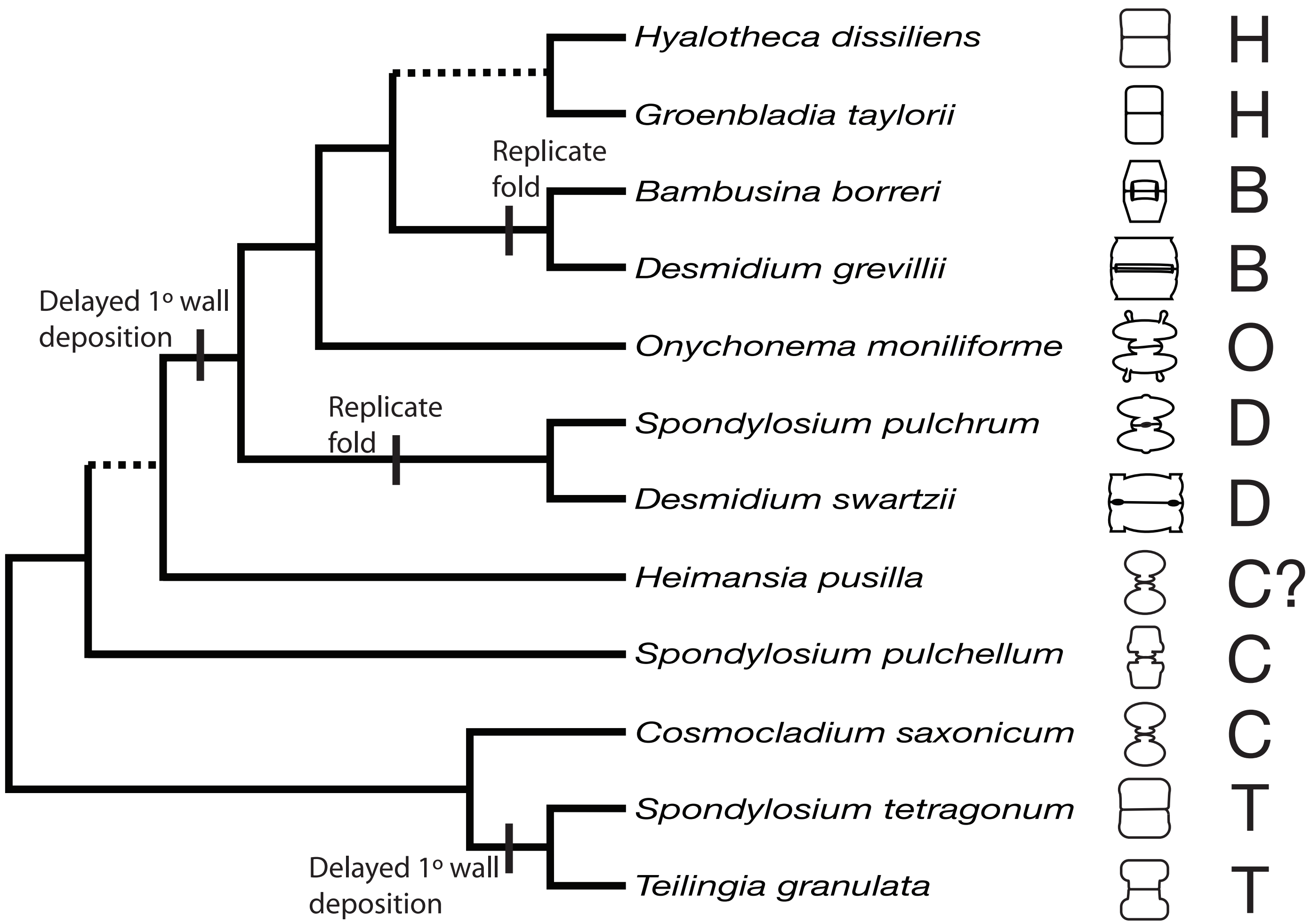


Figure 4.4 Model cladogram showing distribution of cell division syndromes. Model based on the Maximum Likelihood topology with representative taxa. A diagnostic stage of cell division is shown on the right. Letters refer to the general type of cell division: B is the *Bambusina*-type, C is the *Cosmarium*-type, D is the *Desmidium*-type, H is the *Hyalotheca*-type, O is the *Onychonema*-type and T is the *Teilingia*-type. Characteristics shared by a lineage, such as a delay in primary wall deposition, are indicated on the model. Branches that are not strongly supported in the ML phylogeny are dashed.

Chapter 5. Systematic revision of some filamentous Desmidiaceae (Zygnematophyceae, Charophyta)

Abstract

Molecular phylogenetic and ontogenetic investigations of filamentous Desmidiaceae (Zygnematophyceae) indicated that some genera in this family are not monophyletic but that the discovered clades share structural and developmental synapomorphies. To bring the taxonomy, phylogeny and structure of these organisms into synchrony I propose a number of taxonomic changes. I have emended the genus *Desmidium*, and resurrected the genus *Didymoprium*. I also propose moving two species of *Spondylosium* to other genera, one to *Desmidium pulchrum* and the other to the newly created genus *Isthmocatena*. The characteristics of these newly circumscribed taxa are discussed as well as the systematic value of various characteristics for predicting relatedness in the filamentous Desmidiaceae.

Introduction

Desmids are a group of mostly unicellular microalgae that are known for their structural diversity. Many thousands of species are thought to inhabit freshwater and semi-terrestrial habitats on every continent. Although overshadowed by their unicellular relatives, a number of filamentous species exist, including the namesake *Desmidium*.

Most filamentous genera contain fewer than fifty species (Gerrath, 1993). However, these genera are structurally diverse, being distinguished by the size and shape of their processes, the presence or absence of granules, and the distribution of pores (Croasdale et al., 1983). Previous investigations discovered some differences in the process of cell division as well (Hauptfleisch, 1888; Gerrath, 1973; Krupp and Lang, 1985a) (Chapter 4). In molecular phylogenetic analyses most of the genera were found to form a monophyletic group to the exclusion of genera of unicellular taxa and the filamentous *Phymatodocis nordstedtiana* (Gontcharov et al., 2003) (Chapter 4). Previous studies included few representatives of each genus and, with the exception of Chapter 4, did not address the correlative structural differences and synapomorphies of the groups found.

Most unicellular Desmidaceae use the *Cosmarium*-type cell division (Pickett-Heaps, 1972), as do some filamentous and colonial species (*Spondylosium pulchellum*, and probably *Heimansia pusilla* and *Cosmocladium saxonicum*). This type of cell division involves the deposition of primary wall material before a stage of substantial cellular elongation. The new walls then expand to form the other half of the cell and secondary wall is deposited when the cell is almost full sized, at which point the primary wall is dehisced (Pickett-Heaps, 1972). Other filamentous species use simple centripetal cell division (e.g., *Hyalotheca*), centripetal cell division with delayed formation of the separating wall (e.g., *Teilingia* and *Spondylosium tetragonum*), or some combination of a division vesicle and the deposition of cylinders of cell wall material (e.g., *Onychonema*, *Desmidium* and *Bambusina*) (Chapter 4).

Studies of cell division indicate that the desmids are in fact structurally and developmentally diverse. Because of this structural diversity, taxonomy of desmids is particularly difficult. Desmidologists maintain a complex classification of varieties and forms. Characteristics of varieties are thought to be heritable and these varieties are, generally, thought to be incapable of interbreeding. Characteristics of formae are thought not to be heritable but may be induced by environmental factors. Disagreement among authors as to which characteristics are heritable and which are not as well as which characteristics are sufficient for species differentiation has led to a great deal of shuffling among the different ranks and, consequently, to a high degree of synonymy. An additional peculiarity of desmid taxonomy is that nomenclature of desmids has a starting point (Ralfs, 1848) later than that of most other algae, which is Linneaeus's "Species Plantarum" (1753). It was thought that Ralfs accounted for every desmid taxon reported at that time. As a result, all names of desmids published before 1848, including those published by Ralfs, were devalidated (Art. 13.1e Vienna Code, McNeill et al., 2006).

In this study, we increased taxon sampling among the filamentous Desmidiaceae, compared to previous studies, to determine the phylogenetic relationships of as many species as possible. Because of previous studies, we hypothesized that the molecular phylogenetic relationships would be inconsistent with the traditional classification. In order to better predict relationships of unsampled species, we collected structural data including chloroplast shape and modes of cell division for the investigated taxa. The taxonomic implications are discussed and, in some cases, changes to the existing nomenclature are proposed.

Materials and Methods

Strains used in this study were requested from culture collections or isolated from the wild. A list of strains, their collection information and GenBank accession numbers is provided in Table 5.1. All strains were maintained in Guillard's Woods Hole Medium or Bold's Basal Medium (Nichols, 1973) enriched with 20 mL soil extract per liter of medium and kept in a growth chamber at 18º C under fluorescent lights with 30 µE flux.

Strains were identified using Croasdale et al. (1983). DNA extraction and PCR amplification followed previously reported methods (Chapter 3).

Fragments of the chloroplast *rbcL* and mitochondrial *cox* III genes were analyzed separately and then combined into a single dataset of 1962 characters and 60 taxa. Under the Maximum Parsimony criterion (MP), 100 random addition sequences were used in a heuristic search for the shortest tree in Paup* v. 4b10 (Swofford, 2003). Bootstrap support (BS) was estimated from 500 pseudoreplicates with 10 random addition sequences per pseudoreplicate. Under the Likelihood criterion (ML), the GTR+I+G model was used with ten random addition sequence replicates and TBR branch swapping in the heuristic search. One hundred bootstrap pseudoreplicates were generated in PhyML (Guindon and Gascuel, 2003) with the GTR model, 6 rate categories, and the invariant sites and gamma distribution estimated from the data. Bayesian Inference (BI) was also employed as implemented in MrBayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003). Four chains run for four million generation (sampled every 100) with the first 2501 trees discarded as burnin were used to estimate the posterior probabilities (PP). Two independent BI analyses converged on the same topology.

In addition to molecular analysis, strains were investigated microscopically. Observations were made by DIC light microscopy on a Zeiss Axioskop compound microscope (Zeiss, Germany). Digital photomicrographs of the species were recorded using a Zeiss AxioCam LCD camera (Zeiss, Germany). To observe cell wall structure, cells were fixed in 3.5% glutaraldehyde for 1 hour and then dehydrated in an ethanol series. To observe timing of cell wall deposition, cells were fixed and then rinsed three times in tap water, stained with 1% Calcofluor White for one hour and then rinsed twice in tap water. Stained cells were observed on the same microscope but were illuminated with a broad-band mercury arc lamp.

Table 5.1 Strains investigated

Taxon	Strain	*rbcL*	*cox* III	Location
Bambusina borreri	JH0125	EF371284	EF371071	Lake Tomohawk, WI
Bambusina borreri	JH0199			Oxbow Lake, WI
Closterium acerosum	UTEX 1075	EF371285	EF371072	IN
Closterium ehrenbergii var. *malinvernianum*	JH0013	EF371286	EF371073	Lake Artemisia, MD
Closterium libellula	JH0021	EF371287	EF371074	Jyme Bog, WI
Desmidium aptogonum	JH0385			Caroline Co., MD
Desmidium aptogonum	JH0387			Caroline Co., MD
Desmidium aptogonum	SVCK 108			Finland
Desmidium aptogonum var. *ehrenbergii*	JH0184	EF371298	EF371085	Lake Tomohawk, WI
Desmidium aptogonum var. *ehrenbergii*	JH0188		----------	Lake Tomohawk, WI
Desmidium baileyi	JH0155			Bird Lake Road Bog, WI
Desmidium baileyii	JH0228	EF371299	EF371086	Spencer Lake, WI
Desmidium grevillei [cylindricum]	SVCK 113			Finland
Desmidium grevillei	JH0094			Wake Co., NC
Desmidium grevillei	JH0096			Washington Co., OH
Desmidium pseudostreptonema	JH0482			Thailand
Desmidium pseudostreptonema	JH0513			Thailand
Desmidium swartzii var. *amblyodon*	JH0042	EF371297	EF371084	Lake Artemisia, MD
Desmidium swartzii var. *amblyodon*	JH0112			Jyme Bog, WI
Desmidium swartzii var. *amblyodon*	JH0121			Bug Lake, WI
Desmidium swartzii var. *amblyodon*	JH0136			Hemlock Lake, WI
Desmidium swartzii var. *amblyodon*	JH0195			Oxbow Lake, WI
Desmidium swartzii var. *swartzii*	JH0122			Bug Lake, WI
Desmidium swartzii var. *swartzii*	JH0150			Bird Lake Road Bog, WI
Desmidium swartzii var. *swartzii*	JH0231			Oneida Co. WI
Gonatozygon monotaenium	UTEX 1253	U71438	EF371092	MN
Gonatozygon pilosum	ACOI 1096	EF371303	EF371093	Serra da Estrela, Portugal
Groenbladia taylorii	JH0339			Spencer Lake, WI
Heimansia pusilla	SVCK 428	EF371291	EF371078	Deming Pond, MN
Hyalotheca dissiliens	JH0187		----------	Lake Tomohawk, WI
Hyalotheca dissiliens	SAG 384-2	AF203499	EF371095	?
Hyalotheca mucosa	JH0003			Tucker Co., WV
Hyalotheca mucosa	JH0055	EF371305	EF371096	Washington Co., OH
Hyalotheca mucosa	JH0063			Washington Co., OH
Hyalotheca mucosa	JH0415			Caroline Co., MD

Micrasterias foliaceae	NIES 297	EF371311	EF371102	Hiroshima, Japan
Micrasterias radiata	JH0064	EF371313	EF371104	Washington Co., OH
Micrasterias rotata	UTEX 1941	EF371312	EF371103	Beaver Lake, BC, Canada
Onychonema laeve var. *micracanthum*	JH0198	EF371318	EF371111	Oxbow Lake, WI
Onychonema laeve var. *micracanthum*	JH0266			Bird Lake Road Bog, WI
Onychonema moniliformis	JH0420		----------	Caroline Co., MD
Penium margaritaceum	UTEX 600	EF371321	EF371114	?
Penium spirostriolatum	SVCK 205	EF371324	EF371117	Hamburg, Germany
Phymatodocis nordstedtiana	JH0164	EF371325	EF371119	Bird Lake Road Bog, WI
Phymatodocis nordstedtiana	SAG 47.89	AJ553962_1	EF371118	Nacogdoches, TX
Spondylosium pulchellum	JH0368			Spencer Lake, WI
Spondylosium pulchellum	SVCK 365	AF203505	EF371137	Ireland
Spondylosium pulchrum	JH0269	EF371341	EF371138	Bird Lake Road Bog, WI
Spondylosium tetragonum	SVCK 440	AF203498	EF371094	Deming Pond, MN
Staurastrum arctiscon	JH0014	EF371343	EF371140	Lake Artemisia, MD
Staurastrum polytrichum f. *biseriatum*	JH0015	EF371344	EF371141	Lake Artemisia, MD
Teilingia granulata	JH0140	EF371351	EF371148	Hemlock Lake, WI
Teilingia granulata	SAG 25.88		----------	Mira, Portugal
Teilingia granulata	SAG 39.83	EF371335	EF371130	Neustift, Austria
Teilingia granulata	UTCC 284	AF203504	EF371131	Baby Lake, ON, Canada
Teilingia granulata [Spondylosium planum]	SVCK 418		----------	Lake Mucubaj, Venezuela
Teilingia granulata [Spondylosium secedens]	SVCK 24		----------	Husum, Germany

Notes: ACOI, Coimbra Collection of Algae; NIES, National Institute for Environmental Studies; SAG, Sammlung von Algenkulturen der Universität Göttingen; SVCK, Sammlung von Conjugaten-Kulturen; UTEX, Culture Collection of Algae at University of Texas; UTCC, University of Toronto Culture Collection of Algae and Cyanobacteria. Vouchers of strains beginning with JH are available from the authors. Taxa in brackets were incorrectly identified in culture collections.

Results

Likelihood analysis of the *cox* III fragment revealed a single lineage of filamentous and colonial species (87/92/.96; MP, ML, PP). As in previous analyses (Gontcharov et al., 2003; Chapter 3), the filamentous species were split into two major clades, one containing *Teilingia granulata*, *Spondylosium tetragonum*, *Cosmocladium saxonicum* and two strains of *Hyalotheca mucosa* (analogous to Figure 5.1); and the other containing the remaining filamentous species. *Spondylosium* spp. were found in three different strongly supported clades. *Hyalotheca* spp. were also found in two different clades, one sister to *Groenbladia taylorii* and the other resolved separately. *Desmidium grevillei* was sister to *Bambusina borreri* and resolved separate from other *Desmidium* spp.. Relationships among species of *Desmidium* were generally poorly resolved, although clades of *D. baileyi* (100/100/1.0), *D. pseudostreptonema* (99/100/1.0) and *D. swartzii* var. *swartzii* (83/97/1.0) were resolved with high support. *Desmidium aptogonum*, *D. aptogonum* var. *ehrenbergii*, and *D. swartzii* var. *amblyodon* were found to share similar primary sequences (Figure 1).

Likelihood analysis of the *rbcL* dataset recovered a similar topology, although the (*Teilingia, Cosmocladium*) clade was sister to a clade of unicellular desmids with very little support (-/-/-). Additionally, the relative positions of *Heimansia pusilla* and *Spondylosium pulchellum* were reversed with respect to the *cox* III and 2-gene dataset (Figure 5.1). The clade of *Desmidium aptogonum*, *D. aptogonum* var. *ehrenbergii* and *D. swartzii* var. *amblyodon* showed some unexpected strongly supported pairings including: *D. aptogonum* JH0387 with *D. swartzii* var. *amblyodon* JH0042 and JH0136 (96/97/1.0) and *D. swartzii* var. *ambylodon* JH0112 with *D. aptogonum* JH0385. The gene fragments from *D. aptogonum* JH0385 and JH0387 were amplified and sequenced a second time and found to be correct.

In spite of the strongly supported differences among *Desmidium* spp., most relationships were congruous between datasets, so the two genes were concatenated into a single dataset and analyzed again. The estimated phylogeny was most similar to the *cox* III and the areas of conflict between the *cox* III and *rbcL* datasets were not strongly supported (i.e. the placement of *Heimansia pusilla* (-/-/.71) and the relationships among strains in the *Desmidium aptogonum*, *D. swartzii* var. *amblyodon* clade (Figure 5.1). Based on this dataset, the filamentous forms are monophyletic with moderate support (67/84/1.0) and the two major lineages received stronger support than in the single-gene analyses. Results of the morphological investigation are summarized in Figure 5.2.

Discussion

Phylogenetic analyses often disagree with traditional systematic treatments. In the case of the conjugating green algae, previous systematic treatment relied almost exclusively on structural characteristics of the cell walls (Croasdale et al., 1983), although a few treatments considered chloroplast shape (Carter, 1919b, 1919a; Teiling, 1952). My analysis combined observations on the cell shape, chloroplast and cell division characteristics of the organisms with a molecular phylogenetic approach and revealed congruence between the molecular phylogeny and the biology and morphology of the organisms.

Strains of *Spondylosium* were discovered in three distinct lineages (Figure 5.1). This result is supported morphologically by the fact that these organisms have different modes of cell division (Chapter 4) (Figure 5.2). I recognize three developmentally dissimilar organisms hat were previously placed in a single genus. These species share a number of characteristics with their sister taxa (Figure 5.2). *Spondylosium tetragonum* undergoes cell division much like *Teilingia granulata* and can be structurally almost indistinguishable under certain environmental conditions. *Spondylosium pulchrum*, shares a mode of cell division very similar to that of *Desmidium swartzii*. *Spondylosium pulchellum* utilizes the *Cosmarium*-type cell division that is, most likely, plesiomorphic.

Desmidium sensu lato is not monophyletic: *D. grevillei* is sister to *Bambusina borreri*. The phylogeny is partially consistent with Ralfs' (1848) systematic treatment, in which *Desmidium grevillei* and *Bambusina borreri* were grouped in *Didymoprium*. Ralfs (1848) noted not only the differences in cell shape, but also the differences in the chloroplast shape, stating that in *Desmidium* (ie. *D. swartzii*), the number of lobes of the chloroplast corresponded to the number of angles of the cell, while in *Didymoprium* (which is oval, not angular, in apical view), this was not the case. Based on these data, that assertion is accurate. *Desmidium swartzii* and its kin can be distinguished from *D. grevillei* and *Bambusina* spp. by the shape of the chloroplast: *Desmidium swartzii* having bifurcate lobed chloroplasts where each lobe extends from a central mass into the angles of the cell, and *Desmidium grevillei* having an irregular, radiating lobed chloroplast, often with four lobes but sometimes more and never bifurcating, much like *Bambusina borreri*. In fact, the chloroplasts of *D. grevillei* are very much like those of *Hyalotheca*. These two genera can be difficult to distinguish in field specimens, but careful observation of cell division should provide ample evidence of their identity.

The discovery of two lineages of *Hyalotheca* spp. was initially dismissed as contamination, but repeated extractions resulted in identical sequences. Isolation of a second strain with similar phylogenetic affinities from a geographically different location suggests that this is not a contaminant but a biological or analytical artifact. I cannot, however, offer any quantitative structural differences between these strains of *Hyalotheca mucosa* and other strains allied to *H. dissiliens*. (Figure 5.1). Further investigation may reveal structural, physiological or developmental disparities.

The fact that relationships among three varieties of *Desmidium* (*D. aptogonum*, *D. aptogonum* var. *ehrenbergii* and D. *swartzii* var. *amblyodon*), were unresolved was not unexpected. In single gene datasets, most of the strains in this group shared very similar primary sequences and the greatest difference between any two of these strains in the combined dataset was 22 nucleotides (about 1%). It may be that these strains represent a species complex capable of interbreeding, but it may also be that the molecular markers used are too conserved to resolve these relationships. The molecular markers used are often employed to estimate relationships among families and genera of angiosperms (Bell et al., 2005; Yoo et al., 2006). The relationships are not resolved and the apparent patterns are likely the result of lineage sorting. Other, faster evolving molecules would have to be sampled in order to test this hypothesis.

In the case of filamentous Desmidiaceae, overall similarity of the organisms, particularly among *Desmidium* spp. and *Spondylosium* spp., has masked the underlying biological differences. Cell division is one of the most important cellular functions and species with three different kinds of cell division were assigned to *Spondylosium*. While gross structural characteristics may be sufficient for identification, developmental differences may be hidden just below the intricately ornamented walls. Basic developmental and cytological characteristics of most species of conjugating green algae are unknown. Authors rarely record chloroplast shape or pyrenoid position, much less aspects of cell division. Future systematic investigations would benefit greatly from careful observation of the organisms. By combining careful morphological observation and molecular phylogenetic data, it is possible to achieve a greater understanding of systematic relationships among these complex organisms.

Systematic Revisions

Evidence from my study and previous studies suggests that three currently recognized genera are polyphyletic, resulting in nomenclatural ambiguities. Because of the strong agreement between the molecular phylogeny and the morphology of certain groups of filamentous Desmidiaceae, I venture to propose changes to an already difficult nomenclature.

The proposed revisions center on the classification of three organisms: *Desmidium grevillei*, *Spondylosium pulchrum*, and *S. pulchellum*. It has been shown that *Desmidium grevillei* is only distantly related phylogenetically to other *Desmidium* species (Gontcharov et al., 2004; Figure 5.1). *Desmidium swartzii* was designated the type of the generic name *Desmidium* by Nägeli (1849) so the generic name resides with the clade containing that species. The phylogenetic relationship between *Bambusina borreri* and *Desmidium grevillei* gives some taxonomic leeway in the generic assignment. One could reunite *Desmidium grevillei* and *Bambusina borreri*, thus reconstituting *Didymoprium* as circumscribed by Ralfs (1848). However, only a single species of *Bambusina* and *Desmidium grevillei* were available for morphological and molecular phylogenetic analysis and it would be premature to speculate on the phylogenetic placement of other species. In light of this uncertainty, I choose to resurrect the generic name *Didymoprium* for *D. grevillei* and maintain *Bambusina* as a separate genus. Further revision may be required as strains of *Bambusina* and *Didymoprium* are found and investigated.

Spondylosium pulchrum was originally described as a species *Sphaerozosma*, but was later assigned to *Spondylosium* because of the absence of apical processes typical of the genus *Sphaerozosma*. The species is, however, rather unlike any other species of *Spondylosium*: it is much larger and uses the *Desmidium*-type cell division (Chapter 4) (Figure 5.2). Because its mode of cell division and chloroplast shape agree with *Desmidium* species, and its phylogenetic placement within the *Desmidium* clade is strongly supported (100/100/1.0), I propose to move this taxon to the genus *Desmidium*, as *Desmidium pulchrum* (Bailey ex Ralfs) J. D. Hall *comb. nov.*.

Although the exact phylogenetic position of *Spondylosium puchellum* is not strongly supported (-/70/*), it is clearly distinct from all other species of the genus for which I have molecular data. I have no molecular data for *S. depressum*, the lectotype of the generic name designated by Croasdale et al. (1983). This species is not known from North America is rarely reported from Europe. I have not seen any material that could be designated as the type material for that species. One of the first published images of the species showed a short filament with a single dividing cell (Pritchard, 1861, pl. III, fig. 9). This figure shows a simple wall across an elongated isthmus indistinguishable from that which has been reported for *Teilingia granulata* and *S. tetragonum* (Figure 5.2). While *S. depressum* cannot conclusively be assigned to any group, the figure is inconsistent with the mode of cell division reported for *Desmidium pulchrum* (Bailey ex Ralfs) J. D. Hall *comb. nov*. (*Spondylosium pulchrum*) and *Spondylosium pulchellum* (Chapter 4) (Figure 5.2).

Because the existing figures of *Spondylosium depressum* indicate that it has a mode of cell division unlike that of *S. pulchellum*, I propose to assign a different generic name to *Spondylosium pulchellum, Isthmocatena pulchella* (Archer) J. D. Hall *comb nov.*. This new genus differs from the genus *Spondylosium* in its mode of cell division, general shape of the cell and the deep constriction at the isthmus. It differs from the structurally similar cells of *Euastrum* and *Cosmarium* in that it forms filaments. It is unknown if other species currently assigned to *Spondylosium* are closely related to this species, but it seems very likely that some species of *Cosmarium* or *Euastrum* may be.

These taxonomic changes resolve phylogenetic incongruence with the systematic treatment in the case of *Desmidium* and *Spondylosium*, however *Hyalotheca mucosa* was also shown to be polyphyletic. I defer taxonomic changes in this case until I have some indication of the structural differences between the groups (if there are any), and to which group the name *Hyalotheca* should be assigned. Croasdale et al. (1983) designated *Hyalotheca mucosa* as the type of the generic name. Strains identified as *H. mucosa* were resolved in two different clades. It is possible that the apparent phylogenetic placement of *Hyalotheca* strains is an artifact of contamination, model misspecification or some more complex evolutionary process such as organellar capture. The cultures appear to be free of contamination, and a similar relationship was reported by Gontcharov et al. (2003) for a different strain of *Hyalotheca*, creating an interesting if difficult conundrum.

I had hoped to designate physical specimens as lectotypes for the three species here treated. Suitable material was not found. If specimens identified by original authors were found, it would be appropriate to designate these as additional lectotypes. Although I am hesitant to designate any of my strains, or those from culture collections as epitypes because none are from the type localities, I believe all strains treated here are correctly identified and their structural characteristics are consistent with the names assigned.

***Desmidium* Agardh ex Ralfs**

Cells form filaments connected by apical processes; normally broader than long; in apical view, cells oval, triangular, or multiangular; in apical view, chloroplasts lobed and bifurcate with one lobe extending into each angle of the cell; cell division involves formation of a division vesicle as well as the deposition of a cylinder of cell wall material at each apical process before a final period of elongation. Genus differs from *Didymoprium* and *Bambusina* in that the cells connect by apical processes, that the number and position of division cylinders correlates to the apical processes, and that the chloroplast is both bifurcate and the lobes extend into the angles of the cells.

Desmidium pulchrum* (Bailey ex Ralfs) J. D. Hall *comb. nov.

Basionym = *Sphaerozosma pulchrum* Bailey ex Ralfs 1848 Brit. Desmid.: 209
Synonym = *Spondylosium pulchrum* (Bailey ex Ralfs) W. Archer 1861: 724
Lectotype here designated = Ralfs 1848 Brit. Desm.: 209, pl. 35, fig. 2a, 2b
Type location = West Point, NY; Princeton, NJ

Comment. - Excludes *Sphaerozosma pulchrum* var. *planum* Wolle (1892 Desmids U.S.: 29, pl. 60, fig. 3,4) and *Spondylosium pulchrum* var. *planum* (Wolle) W. West & G. S.

West (1896 Trans. Lin. Soc. London, Bot. 5:231). Material held at BM (slide 11798) that was sent to Ralfs by Bailey was in too poor condition to designate as a type specimen, leaving only the image drawn by Ralfs as a possible lectotype.

Didymoprium **Kütz. ex Ralfs**

Cells form filaments connected along the apex; cells broader than long; in apical view, cells circular or somewhat elliptic, often with two small protrusions on opposite sides of the cell; in apical view, chloroplasts lobed (usually four or more) without bifurcations, chloroplast radiate from a central mass; cell division involving deposition of a single central cylinder of cell wall material (much like *Bambusina borreri*) without an inflated division vesicle. Differs from *Bambusina* in that the cells are broader than long and from *Hyalotheca* in that cell division involves the deposition of a cylinder of cell wall material.

Type species designated here = *Didymoprium grevillei* Ralfs 1848 Brit. Desm.: 57

Didymoprium grevillei **Ralfs 1848**

Synonym = *Desmidium cylindricum* Grev. 1827 Scott. Crypt. Fl. 5; Pl. 293 - devalidated
Synonym = *Arthrodesmus cylindricus* Menegh. 1840 Linnea 14: 204 - devalidated
Synonym = *Desmidium compressum* Corda 1840 Alman. Carlsbad 10: 203 - devalidated
Synonym = *Didymoprium grevillii* Kütz. 1843 Phyc. Generalis: 166 (?) - illegitimate
Synonym = *Didymoprium cylindricum* (Grev.) Ralfs 1845 Ann. & Mag. Nat. Hist. 16: 10 - devalidated
Synonym = *Hyalotheca grevillii* Bréb. 1846 – devalidated
Type here designated = Ralfs 1848 Brit. Desm. 57, pl. 2, fig. a-k
Type location here designated = Prussia; Prague; Carlsbad; Reichenberg; Falaise; New York, Rhode Island, USA

Comment: This species was described by Greville (1827) as *Desmidium cylindricum*. Kützing (1843) later moved it to his new genus *Didymoprium*, but in so doing unnecessarily changed the epithet to *grevillei* [*grevillii*]. Ralfs (1845) restored the original epithet, naming the species *Didymoprium cylindricum* (Grev.) Ralfs, but in his starting-point monograph (Ralfs, 1848), he adopted Kützing's epithet. Because this monograph is the start date for desmid taxonomy, and Kützing's previous combination was invalid, Ralfs (1848) is the appropriate authority for the taxon *Didymoprium grevillei*. Attempts to locate Greville's materials were unsuccessful and I here designate the image from Ralfs (1848) as the lectotype of this species.

Isthmocatena **J. D. Hall gen. nov.**

Diagnosis: Genus novum Desmidiacearum. Cellulae plerumque catenaformes, raro solae; sinus profundus; semicellulae trapeziformes compresses; ad regionem isthmum paululum inflatae; a vertice visae ellipticae; divisio a *Cosmarium* similis.

Cells forming short filaments, sometimes unicellular; individual cells deeply constricted, hemicells trapeziform and compressed; somewhat lobed near the isthmus; ovoid in apical view; surface finely punctate; chloroplast 4-lobed with the lobes extending from a mass above the isthmus toward the apex of the cell appearing furcated in face and apical view; cell division via the *Cosmarium*-type cell division.

Etymology. Name refers to the median constriction found in these cells, and their tendency to form short filaments.

Type species: *Isthmocatena pulchella* (Archer) J. D. Hall comb. nov.

***Isthmocatena pulchella* (Archer) J. D. Hall comb. nov.**
Basionym = *Sphaerozosma pulchellum* W. Archer 1858 Nat. Hist. Rev. 5 (Proc.): 253, pl. XXI: fig. 7
Synonym = *Spondylosium pulchellum* (W. Archer) W. Archer 1861 History of Infusoria: 724
Synonym = *Sphaerozosma secedens* var. *pulchellum* (W. Archer) Hansgirg 1888 Prodr. Alg. Bohmen 1: 170
Type location here designated = Near Dublin, Ireland
Lectotype here designated = Archer 1858 Nat. Hist. Rev. 5 (Proc.): 253, pl. XXI: fig. 7 as *Sphaerozosma pulchellum* W. Archer

Comment. – I was not able to locate material from Archer's collections that corresponded to this taxon. I designate the original drawing as the lectotype.

***Bambusina borreri* (Ralfs) Cleve** 1864 Öfvers. Forh. Kongl. Svenska Vetenskaps-Akad. 20: 496

This taxon should be added to the list of conserved taxa as it is the type for the conserved genus *Bambusina* Cleve 1864 l.c.

Table 5.2 List of names and synonyms

Arthrodesmus Ehrenb. ex Ralfs
A. cylindricus (Grev.) Menegh. (devalidated)
Bambusina Kütz.
B. borreri (Ralfs) Cleve
Cosmarium Corda ex Ralfs
Cosmocladium Bréb.
C. saxonicum de Bary
Desmidium C. Agardh ex Ralfs
D. aptogonum (Ralfs) W. Archer
D. aptogonum var. *ehrenbergii* Kütz.
D. baileyi (Ralfs) Nordst.
D. compressum Corda (devalidated)
D. cylindricum Grev. (devalidated)
D. grevillei Ralfs
D. pseudostreptonema West & G.S. West
D. pulchrum (Bailey ex Ralfs) J.D. Hall
D. swartzii Ralfs
D. swartzii var. *amblyodon* (Itzigs.) Rabenh.
Didymoprium Kütz. ex Ralfs
D. cylindricum (Grev.) Ralfs (devalidated)
D. grevillei Kütz. ex Ralfs
Euastrum Ehrenb. ex Ralfs
Groenbladia Teiling
G. taylorii A.M. Scott & Grönblad
Heimansia Coesel
H. pusilla (Hilse) Coesel
Hyalotheca Kütz. ex Ralfs
H. dissiliens Ralfs
H. grevillei Bréb. (devalidated)
H. mucosa Ralfs
Isthmocatena J.D. Hall
I. pulchella (W. Archer) J.D. Hall
Onychonema G.C. Wall.
Phymatodocis Nordstedt
Phymatodocis nordstedtiana Wolle
Sphaerozosma Corda ex Ralfs
S. pulchrum Bailey ex Ralfs
S. pulchrum var. *planum* Wolle
Spondylosium Bréb. ex Kütz.
S. depressum Bréb. ex Kütz.
S. pulchellum (W. Archer) W. Archer
S. pulchrum (Bailey ex Ralfs) W. Archer
S. pulchrum var. *planum* (Wolle) West & G.S. West
S. tetragonum West
Teilingia Bourr.
Teilingia granulata (J. Roy & Bisset) Bourr.

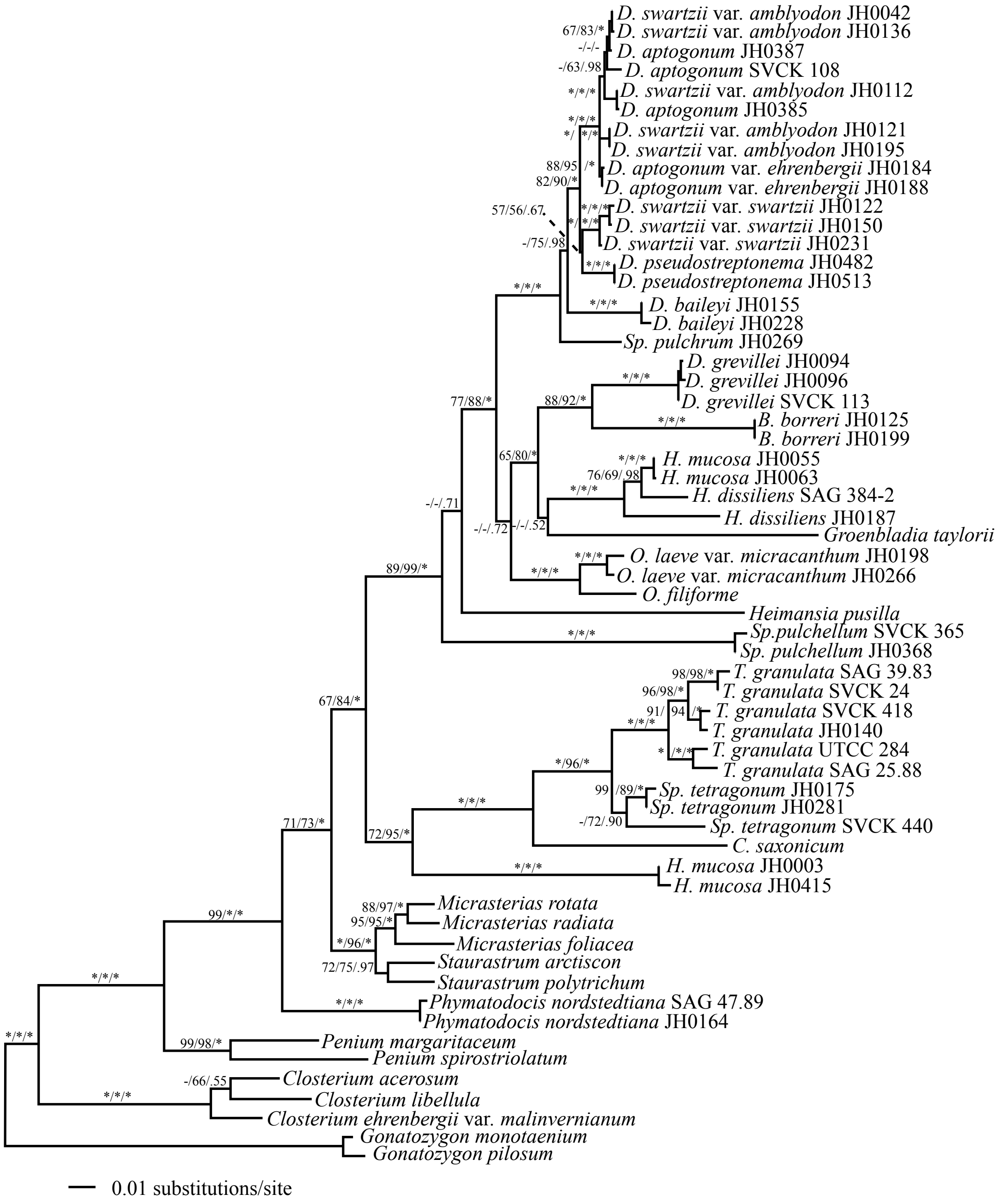
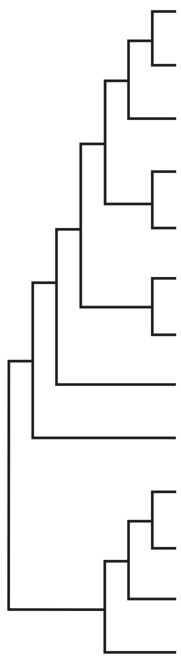


Figure 5.1. Phylogeny of the filamentous Desmidiaceae based on the ML tree from the *rbcL* and *cox* III combined analysis. Numbers above the branches are MP and ML BS and BI PP, respectively. Support of 100 (BS) and 1.0 (PP) are indicated with an asterisk and support of less than 50 (BS) or less than 0.50 (PP) are indicated with a dash.



	Shape	Symmetry	Division	Vesicle	Delay	Plastid	Connection
Hyalotheca dissiliens	Circular	Radial	*Hyalotheca*	No	Yes	Lobed, Radial	Apex
Groenbladia taylorii	Circular	Radial	*Hyalotheca*	No	Yes	Taeniform	Apex*
Onychonema laeve	Oval	Biradiate	*Onychonema*	Yes	Yes	H- Lobed	Apex
Desmidium grevillei	Circular	Radial	*Bambusina*	No	Yes	Lobed, Radial	Apex*
Bambusina borreri	Circular	Radial	*Bambusina*	No	Yes	Lobed, Radial	Apex
Spondylosium pulchrum	Oval	Biradiate	*Desmidium*	Yes	Yes	Lobed, Bifurcate	Process
Desmidium swartzii	Angular	Radial	*Desmidium*	Yes	Yes	Lobed, Bifurcate	Process
Heimansia pusilla	Oval	Biradiate	*Cosmarium*	No	No	H-Lobed (?)	Primary**
Spondylosium pulchellum	Oval	Biradiate	*Cosmarium*	No	No	H-Lobed	Apex*
Teilingia granulata	Oval	Biradiate	*Teilingia*	No	Yes	H-Lobed	Apex*
Spondylosium tetragonum	Oval	Biradiate	*Teilingia*	No	Yes	H-Lobed	Apex*
Cosmocladium saxonicum	Oval	Biradiate	*Cosmarium*	No	No	H-Lobed (?)	Pore thread
Hyalotheca mucosa	Circular	Radial	*Hyalotheca*	No	Yes	Lobed, Radial	Apex*

Figure 5.2 Structural characteristics of species investigated. Shape = cell shape in apical view. Cells which are circular are generally subcircular or broadly oval, angular species may be 2 to 4-angled depending on the species; Symmetry = symmetry of cell contents in apical view; Division = division type as described by Hall et al. 2007a; Vesicle = division involves formation of an inflated division vesicle; Delay = delay in deposition of primary wall material with respect to the *Cosmarium*-type cell division; Plastid = plastid shape, H-lobed means that the chloroplast lobes extend from a central mass forming something like an "H" in any view, (?) indicates uncertainty; Connection = point of connection between adjacent cells, all species so far investigated are held together by a shared primary wall of some form.

*Consistent with light microscopical investigation but not confirmed by TEM

**Connected by loosened primary walls (Coesel, 1993)

Chapter 6. Investigating diversity among structurally simple desmids, the Gonatozygaceae (Desmidiales, Zygnematophyceae)

Abstract

The family Gonatozygaceae is the structurally simplest family of desmids. We investigated the phyletic and structural diversity in this family to determine if their structural simplicity masks their overall diversity. We found that phylogenetically distinct lineages of *Gonatozygon kinahanii* and *G. brebissonii* are structurally similar. In the case of *Gonatozygon kinahanii*, these lineages can be distinguished based on traditional taxonomic characteristics, however this was not the case for *G. brebissonii*. We also found that *Genicularia spirotaenia* was embedded in *Gonatozygon* and the two available strains have different structural characteristics. Implications for the systematics and identification of these ecological indicator species are discussed.

Introduction

Before molecular sequence methods were commonly used, most microorganisms were assigned to species based on their physical appearance in the light microscope or their metabolic capabilities (bacteria and some fungi). Investigation of molecular sequence data suggests that many microorganisms have been overlooked by these methods (López-García et al., 2001; Venter et al., 2004). Studies utilizing environmental sequencing methods have detected these novel sequences, but the organisms from which the sequences are derived are entirely unknown. In other cases, strains of known organisms that are structurally similar show an unexpected degree of sequence diversity (Ciniglia et al., 2004). Investigations of molecular sequence diversity are still in their infancy and correlation between these sequences and the organisms from which they are derived has only just begun. Such correlation is necessary if we are to understand the limits of these different measures of diversity. In many cases, organisms that initially seem structurally indistinguishable are found to have cryptic peculiarities, sometimes subtle and other times profound.

Among the eukaryotic algae, the diatoms and the desmids are known for their complexity of unicellular form. However, even among the many thousands of named desmids there are some that are structurally simple. One family in particular, the Gonatozygaceae (Desmidiales, Zygnematophyceae), includes species that are cylindrical and without the processes and lobes characteristic of other desmids. Only about 13 morphological species have been described in the genera *Gonatozygon* and *Genicularia*. Other desmid families contain many more species: Closteriaceae, 140; Desmidiaceae, 3,000 (Gerrath, 1993). Relationships among very few species belonging to the Gonatozygaceae have been investigated using molecular phylogenetic markers (Park et al., 1995). The paucity of structural characteristics makes identification of species difficult. Species in this family are, however, considered positive indicators of water quality (Coesel, 2001).

Previous studies of cell wall structure and molecular phylogeny indicate that the Gonatozygaceae are an ancient lineage of the Desmidiales (Ruzicka, 1970; Mix, 1972; Park et al., 1996; McCourt et al., 2000; Gontcharov, Marin, and Melkonian, 2003) and that the zygnematalean genus *Roya* may be allied to this family (Park et al., 1996; McCourt et al., 2000; Gontcharov et al., 2003). *Gonatozygon* and *Roya* have similar axile chloroplasts, however, the walls of *Roya* are completely without pores or sutures

(Mix, 1972). Species of *Gonatozygon*, the most species-rich genus in the family, are differentiated based on features of the cell wall, shape of the apex of the cells and the cell dimensions (Prescott et al., 1972).

Růžička (1970) concluded that cell size was a poor taxonomic character because the dimensions of many of the varieties were highly variable and sometimes overlapping. Other diagnostic features of the cell shape vary: shape of the apex, the presence or absence of cell wall ornamentation. While this variability can be difficult to assess in wild populations because the variants would be assigned to different species, it is frequently observed in clonal cultures (Hall, personal observation).

With so few structural features and so much variation within those observable features, assessment of identity and diversity becomes difficult. Molecular sequence data are not obscured by structural variability and can sometimes reveal phyletic diversity among structurally simple or homogenous organisms. I hypothesize that because systematic treatments depend heavily on structural characteristics, the small number of species of Gonatozygaceae may be an artifact of the structural simplicity of the cells. To test this hypothesis, I collected sequence data from several genes in 16 strains of *Gonatozygon*, *Genicularia*, and *Roya* including several representatives of each species. I also measured many observable characteristics of the cells including cell wall features, and cell dimensions.

Materials and Methods

Strains were isolated from the wild or requested from public culture collections (Table 6.1). These strains were maintained in either Guillards Woods Hole Medium, or

Bold's Basal Medium enriched with soil water (Nichols, 1973) in a growth chamber at 18º C with cool white fluorescent lights at 30 μE. Cultures were verified and checked for contamination at time of accessioning and when DNA was extracted.

Cells were observed in living condition as well as after several treatments for light and scanning electron microscopy (SEM). Cells were treated to remove extracellular materials and expose the surface of the cell wall. A six-hour treatment of 1% hypochlorite was found to remove the mucilage and bacteria from the surface without noticeably damaging the cell wall. After treatment, cells were rinsed three times in medium to remove the hypochlorite.

Morphological measurements were obtained from approximately 100 individual cells from strains investigated (Table 6.2). All characters traditionally used in systematic treatments were measured. Size measurements were rounded to the nearest hundredth of a micrometer. Width measurements for figure 6.4 and 6.5 were rounded to the nearest micrometer. Length measurements for figures 6.6 and 6.7 were binned into twenty-micrometer groups.

For SEM, cells were first fixed in glutaraldehyde and then dehydrated in an ethanol series. The dehydrated cells were dried in a critical point dryer and mounted with double sided sticky tape and coated with Gold Palladium. Cells were observed at ~5-10 kv on a Hitachi S-4700 FESEM.

DNA was extracted using the Nucleon Phytopure DNA extraction kit and the manufacturer's protocol (Amersham, MA). Three genes, *rbcL*, *psaA* and *cox* III were amplified from the total DNA using published primers (Chapter 3). Sequences were edited in Sequencher 4.0 (GeneCodes) and aligned by eye in MacClade v. 6.0 (Maddison and Maddison, 2000). Outgroup sequences were downloaded from GenBank (Table 6.1).

Each gene was analyzed individually and then concatenated into a single dataset with 4,047 nucleotides. The concatenated dataset was analyzed under the Parsimony (MP) and Maximum Likelihood (ML) criteria in Paup* (Swofford, 2003), PhyML (Guindon and Gascuel, 2003) and under Bayesian Inference (BI) in MrBayes (Ronquist and Huelsenbeck, 2003). MP analysis was performed using a heuristic search with 100 random taxon addition sequence replicates and TBR branch swapping. Five hundred bootstrap pseudoreplicates were used to estimate the support for taxon bipartitions. An optimal ML tree was discovered in Paup* from a heuristic search with three random taxon addition sequence replicates, and TBR branch swapping under the GTR+I+G model with all parameters estimated from the data. Bootstrap analysis was performed in PhyML using the GTR+I+G model with the proportion of invariant sites and the alpha parameter estimated from the data and empirical base frequencies. Markov chains were employed in MrBayes for 4M generations using the GTR+I+G model with the first 2501 trees discarded as burnin. The same topology was recovered in two independent BI analyses.

Table 6.1 Strains investigated and Genbank numbers

Taxon	Authority	Strain	rbcL	psaA	coxIII
Zygnematophyceae					
Desmidiales					
Desmidiaceae					
Bambusina borreri	(Ralfs) Cleve	JH0199	EF371283	EF371177	EF371070
Cosmarium botrytis	(Meneghini) Ralfs	UTEX 301	EF371288	EF371182	EF371075
Desmidium swartzii	Agardh ex Ralfs?	JH0042	EF371297	EF371191	EF371084
Haplotaenium (Pleurotaenium) minutum	(Ralfs) Bando	SVCK 302	EF371326	EF371227	EF371120
Heimansia (Cosmocladium) pusillum	(Hilse) Coesel	SVCK 428	EF371291	EF371185	EF371078
Micrasterias rotata	(Greville) Ralfs	UTEX 1941	EF371312	EF371210	EF371103
Onychonema laeve var. *micracanthum*	Nordstedt	JH0198	EF371318	EF371218	EF371111
Phymatodocis nordstedtiana	Wolle	SAG 47.89	AJ553962_1	EF371225	EF371118
Staurodesmus convergens [Arthrodesmus sp.*]*	(Ehrenberg) Teiling	UTEX 2508	EF371281	EF371175	EF371068
Peniaceae					
Penium cf. *didymocarpum*	Lundell	JH0212	EF371316	EF371216	EF371109
Penium cylindrus	(Ehrenberg) Brébisson ex Ralfs	ACOI 780	EF371320	EF371220	EF371113
Penium margaritaceum	(Ehrenberg) Brébisson ex Ralfs	UTEX 600	EF371321	EF371221	EF371114
Penium spirostriolatum	Barker	SVCK 205	EF371324	EF371224	EF371117
Closteriaceae					
Closterium acerosum	(Schrank) Ehrenberg ex Ralfs	UTEX 1075	EF371285	EF371179	EF371072
Closterium ehrenbergii var. *malinvernianum*	(De Notaris) Rabenhorst	JH0013	EF371286	EF371180	EF371073
Closterium libellula	Focke	JH0021	EF371287	EF371181	EF371074
Spinoclosterium cuspidatum	(Bailey) Hirano	NIES 325	SCU553965	EF371240	EF371132
Gonatozygaceae					
Genicularia spirotaeniae	de Bary	SAG 54.86	GSP553946	EF371197	EF371090
Gonatozygon brebissonii	de Bary	SVCK 210			
Gonatozygon brebissonii	de Bary	JH0375			
Gonatozygon brebissonii	de Bary	JH0377			
Gonatozygon brebissonii var. *laeve*	(Hilse) West & West	JH0033	EF371332	EF371233	EF371126
Gonatozygon kinahanii	(Archer) Rabenhorst	ACOI 350	GK1553945	EF371198	EF371091
Gonatozygon kinahanii	(Archer) Rabenhorst	UTEX 2471			

Gonatozygon kinahanii var. *majus*	Taylor	JH0356			
Gonatozygon monotaenium	de Bary	UTEX 1253	U71438	EF371199	EF371092
Gonatozygon monotaenium	de Bary	SVCK 108			
Gonatozygon monotaenium	de Bary	JH0282			
Gonatozygon pilosum	Wolle	ACOI 1096	EF371303	EF371200	EF371093
Gonatozygon pilosum	Wolle	SVCK 64			
Zygnematales					
Mesotaeniaceae					
Cylindrocystis brebissonii	Meneghini	UTEX 1259	EF371293	EF371187	EF371080
Mesotaenium sp.		JH0031	EF371310	EF371208	EF371101
Netrium digitus	(Ehrenberg) Itzigson & Rothe	UTEX 561	U38698	EF371214	EF371107
Roya anglica	West	UTEX 934	AJ553963	EF371231	EF371124
"Roya anglica"	West	?	U38694	----------	----------
Roya obtusa	(Brébisson) West & West	SAG 168.80	EF371331	EF371232	EF371125
Roya obtusa var. *montanum*	West	JH0357			
Zygnemataceae					
Mougeotia sp.		UTEX 758	AF408252	EF371212	EF371105
Spirogyra maxima	(Hassall) Wittrock	UTEX 2495	DQ15941	EF371244	EF371136
Zygnema cylindricum	Transeau	SAG 689-2	EF371357	EF371262	EF371155
Zygnemopsis minuta	Randhawa	ACOI 60	EF371363	EF371268	EF371161
Zygogonium tunetanum	Gauthier-Lièvre	UTCC 136	AF203509	EF371269	EF371162
Outgroup					
Coleocheatales					
Coleochaete scutata	Brébisson	SAG 3.9	AY082324.1	EF371273	EF371166
Coleochaete nitellarum	Jost	UTEX 1261	AY082325.1	EF371275	EF371168
Coleochaete divergens	Pringsheim	300d1	AY082324.1	EF371272	EF371165
Coleochaete pulvinata	Braun ex Kützing	57b6	AY082307.1	EF371274	EF371167
Coleochaete sieminskiana	Szymanska	10d1	AF408791.1	EF371277	EF371170

Table 6.2 Structural characteristics of some *Gonatozygon* spp.

Taxon	Length (µm)		Width (µm)		Apex (µm)		Pyrenoids		L:W	
G. brebisonii JH0375	119.92	±23.38	6.32	±0.65	4.03	±0.46	5.89	±1.93	19.16	±0.02
G. brebissonii SVCK	114.87	±32.97	6.63	±0.89	4.43	±0.51	5.78	±1.85	17.60	±5.41
G. kinahanii var. *majus* JH0356	291.90	±68.30	19.22	±1.74	NA	NA	11.66	±2.98	15.21	±0.01
G. kinahanii ACOI	180.92	±33.48	12.16	±1.65	11.32	±1.55	6.67	±1.61	15.15	±0.02
G. kinahanii UTEX 2495	526.73	±162.13	14.12	±1.79	13.83	±1.84	13.64	±4.37	37.61	±0.01

Results

Phylogenetic relationships

In the combined ML analysis, *Gonatozygon* and *Genicularia* were recovered as a monophyletic group with very strong support (99/100/1.0; MP/ML/PP) (Figure 6.1). Most *Roya* spp. were found as monophyletic with strong support (100/100/1.0), however, the placement of the *Roya* clade was not supported (-/-/-). The ML tree found in individual gene analyses showed *Roya* sister to the Gonatozygaceae and Peniaceae (*rbcL*, *cox* III) or sister to the Peniaceae and Desmidiaceae (*psaA*), but not as a monophyletic group with the Gonatozygaceae (data not shown). In the combined analysis, the nine most parsimonious trees differed in the placement of *Roya* (never monophyletic with Gonatozygaceae), the placement of *Netrium digitus* and relationships among the Desmidiaceae (data not shown). When all three genes were concatenated, likelihood analyses resolved *Roya* as sister to a clade of Desmidiales excluding Gonatozygaceae with little support (Figure 6.1). *Roya* spp. were found to be monophyletic and *Roya obtusa* and its variety *montana* were sibling taxa (Figure 6.1).

The earliest branching member of the Gonatozygaceae, was *Gonatozygon kinahanii* var. *majus*. This strain was paraphyletic to the typical *G. kinahanii* UTEX 2471, ACOI 350 (Figure 6.1). Additionally, *G. brebissonii* SVCK 210 was found to be paraphyletic with respect to other isolates of that species as well as the variety *laeve*. The dubious strain "*Roya anglica* U38694" appeared sister to a clade of *G. monotaenium* and *G. pilosum*. *Genicularia spirotaenia* was sister to two strains of *Gonatozygon pilosum* with moderate support (76/68/0.93), and included in a larger group with *G. monotaenium* with high support (100/100/1.0).

Structural investigation

Cellular characteristics of living and treated specimens were investigated by light microscopy. Most strains exhibited structural characteristics consistent with published work. The genus *Gonatozygon* is characterized by flat chloroplasts, however, all strains investigated with the exception of varieties of *G. kinahanii*, exhibited a slight ridge down the center of the chloroplast (Figure 6.2a, c). Cells sometimes exhibited several large ridges that extended the length of the chloroplast and appeared similar to the plastids of *Penium* spp., this was particularly true of *G. brebissonii* var. *laeve* (Figure 6.2b). Most species exhibited a series of pyrenoids arranged linearly down the center of the chloroplast (Figure 6.2a, c, f), however, *G. kinahanii* var. *majus* had pyrenoids arranged somewhat irregularly in the chloroplast (Figure 6.2d) as was reported by Taylor (1934). In living specimens, the cell wall of *G. brebissonii* var. *laeve* appeared smooth (data not shown).

A strain designated *Genicularia spirotaenia* SAG 54.86was also included in the study. The cell wall features of this strain were similar to *Gonatozygon monotaenium*. The chloroplast appeared to be either entire or a somewhat reticulate network (data not shown). A second strain, *Genicularia spirotaenia* SVCK 329, was obtained. This strain differed from the included strain at only two nucleotide positions (data not shown). In this strain, however, the chloroplast appeared to be two long chloroplasts (or possibly lobes of a single chloroplast) that joined somewhere near the mid region. Although mostly parietal, the chloroplast crossed the center of the cell (data not shown).

Investigation of the cell walls of the Gonatozygaceae revealed a number of previously unidentified characteristics. Cell walls of *Roya* spp. appeared to be completely without pores or granules (Figure 6.3g). This condition was also true of

Gonatozygon kinahanii and *G. kinahanii* var. *majus* (Figure 6.3d). *G. monotaenium* had small granules (Figure 6.3a). These granules were very short and sometimes acute, appearing as very short spines in living specimens (Figure 6.2a). Granules in *G. monotaenium* are unlike the spines found in *G. pilosum,* which are only slightly longer, but normally attenuated into short spines of irregular length (Figure 6.3b). *Gonatozygon monotaenium* JH0282, differed from the other strains in that the granules were larger and irregularly shaped (visible at the end of the cell in Figure 6.3A). These granules could perhaps be better referred to as verrucae. All strains of *G. brebissonii* had granulate cell walls (Figure 6.2c, 6.3c). However, the granules of *G. brebissonii* var. *laeve* were so fine that the cell walls appeared smooth in living cells (Figure 6.3e). Additionally, treatment with hypochlorite revealed transverse sutures in *G. brebissonii* and *G. brebissonii* var. *laeve* (Figure 6.3e, 6.3f).

In cases where taxa could not be distinguished using structural characteristics, cell size was investigated. Length and width measurements were made in *G. kinahanii* and *G. brebissonii*. Cell width was sufficient to distinguish between the *G. kinahanii* var. *kinahanii* and *G. kinahanii* var. *majus*, (Figure 6.4) but the width distributions in *G. breb*issonii were most overlapping (Figure 6.5) and the two strains had a similar mean width (Table 6.2). Strains could not be distinguished based on cell length (Figure 6.6, 6.7). Cell length was much more variable than cell width, particularly in *G. kinahanii* UTEX 2471.

Discussion

The Gonatozygaceae is the most structurally simple family of desmids.

To test the correlation between structural and genetic diversity, I considered the structural characteristics of the strains in the context of a molecular phylogeny. This combined approach revealed more structural diversity than was previously reported and helped distinguish at least one previously unrecognized lineage of *Gonatozygon brebissonii*. Based on these genes, every strain had unique primary sequence with the exception of two strains of *G. brebissonii* (JH0375, JH0377) collected from the same pond on the same day and two strains of *G. pilosum* (ACOI 1096 and SVCK 64) from Portugal and Norway, respectively. While JH0375 and JH0377 are very likely to be vegetative clones of one another, the strains of *G. pilosum* are less likely to be. Shared primary sequence, in this case, does not necessarily indicate that the organisms are of the same clonal strain. The genes used to estimate the phylogeny are very conservative and may not be informative at the species or subspecies level. The phylogeny does indicate a surprising degree of genetic divergence among strains of *G. brebissonii* and among strains of *G. kinahanii*. This finding supports the hypothesis that the structural simplicity of the group may have resulted in an underestimation of their genetic (and probably species) diversity.

Placement of the Gonatozygaceae among the conjugating green algae is not controversial. Both structural investigation and molecular phylogenetic analyses are consistent with the family being an early branching member of the Desmidiales (Ruzicka, 1970; Mix, 1972; Park et al., 1996; McCourt et al., 2000; Gontcharov et al., 2003). Placement of *Roya* is less certain. Gontcharov et al. (2003) found *Roya* sister to all Desmidiales or part of a clade including *Netrium digitus*. My analyses indicate that *Roya* is part of the Desmidiales to the exclusion of *Netrium digitus* and the best ML tree shows that *Roya* may be more closely related to other Desmidiales than the Gonatozygaceae (Figure 6.1). However, no one placement is strongly supported. The GenBank accession "*Roya anglica*" U38694 is unlikely to be derived from a *Roya* spp. and is certainly not from the strain of *Roya anglica* investigated (as indicated by the information in GenBank). This was previously reported by Gontcharov et al. (2004).

Low bootstrap support for the placement of *Roya* could be the result of biases in the molecular sequence data, biases induced by poor taxon sampling or model misspecification, or could represent a genuine biological phenomenon (e.g., rapid radiation). Bootstrap analyses under the parsimony criterion did not support the placement of *Roya* spp. (data not shown) indicating that model misspecification is an unlikely cause of the ambiguity. While I cannot eliminate the possibility of bias in the genes selected, previous studies also failed to confidently place *Roya* (Gontcharov et al., 2003). Perhaps greater taxon sampling among the basal lineages of the Desmidiales will provide better support for the placement of *Roya*.

Notwithstanding, *Roya* spp. are structurally similar to *Gonatozygon.* They have an axile taeniform chloroplast and their cell wall is smooth like that of *G. kinahanii*. There may be, however, structural or compositional differences in the cell walls. *Gonatozygon* was classified among the Desmidiales because of its cell wall characteristics, namely the presence of an extracellular electron dense matrix that harbors pores, spines and granules (Mix 1972). *Roya* spp. were investigated by light microscopy and SEM and found to lack granules and pores (data not shown).

Among the Gonatozygaceae, phylogenetic analysis revealed a group containing *Gonatozygon pilosum, G. monotaenium* and *Genicularia spirotaenia* as well as paraphyletic assemblages of *Gonatozygon brebissonii* and *G. kinahanii* (Figure 6.1). The identity of the *Genicularia spirotaenia* strain is uncertain as its structural characteristics do not exactly match the description of that species. Interestingly, there is as much nucleotide difference among strains of *Gonatozygon monotaenium* as between *Genicularia spirotaenia* and *Gonatozygon pilosum* (p-dist = 0.0368 vs 0.0283). Perhaps *G. monotaenium* JH0282 represents a different variety or a different species, a hypothesis supported by the difference in the shape of the granules (Table 6.2).

Two lineages of *G. brebissonii* were resolved. Cells of these strains were structurally similar and basic cellular measurements revealed no quantitative differences (Table 6.2). The difference in the mean length (115 µm vs. 120 µm) would probably not be diagnostic in wild material. It is difficult to distinguish these strains structurally, however, they could be distinguished based on nucleotide sequence differences. *Gonatozygon brebissonii* is one of the most common species of the genus and its characteristics are particularly variable (Ruzicka, 1970). It may be that *G. brebissonii* as currently circumscribed includes a number of species. These data also suggest that at least some of these species may be structurally indistinguishable. Many more strains of *G. brebissonii* would be needed to test this hypothesis.

Two strains used in this study were inconsistent with the name assigned to them in public culture collections. *Gonatozygon pilosum* SVCK 64 was assigned to *G. aculeatum* in the culture collection, however, the strain has hairs very similar to *G. pilosum* ACOI 1096 (Figure 6.3b) and not like those of *G. aculeatum* (Figure 6.2e). While it is possible for the hairs of *G. pilosum* to be aculeate, *G. aculeatum* is characterized by a combination of spine shape and size inconsistent with those of strain

SVCK 64. Moreover, this strain was unusual in that fewer than 10% of the cells displayed the expected structural features. Cells taken from the same culture vessel had mostly smooth walls, but a few cells showed both smooth and pilose sections of wall on the same cell indicating that this is a peculiarity of the strain and not a case of a mixture of two different strains.

The second strain, *G. kinahanii* UTEX 2471, was labeled *Mougeotia sp.*. Paradoxically, these two genera are sometimes difficult to distinguish, yet are only very distantly related (McCourt et al., 2000; Gontcharov et al., 2003) (Chapter 3). In this case, the strain can be identified as *G. kinahanii* because adjacent cells in the filament do not share any walls. This is also true of other strains of *G. kinahanii* investigated, and inconsistent with the structure of filaments of *Mougeotia*. Because of the similarity of *G. kinahanii* and *Mougeotia* one must consider the possibility that *G. kinahanii* (or species structurally similar to it) is more common than is reported. It is possible that a number of species currently assigned to *Mougeotia* belong to *Gonatozygon*. If correct, such confusion would require a reevaluation of the use of *Mougeotia* and *Gonatozygon* as indicators of water quality.

In general, structural characteristics were discovered which correlated well with the molecular signatures of the strains investigated. In most cases, these characteristics were sufficient to distinguish taxa. The resolved relationships can also be reconciled with the taxonomy of the strains, except in the case of *G. kinahanii* var. *major*. This species is found to be paraphyletic to the typical variety and could probably be best treated as a species unto itself. The other two varieties, *G. brebissonii* var. *laeve* and *Roya obtusa* var. *montana*, showed some sequence divergence from their respective typical varieties, but their phylogenetic positions are not inconsistent with their nomenclature and no changes are proposed. All characteristics, including cell dimensions, have to be employed to distinguish some strains of *Gonatozygon*. Only in the case of *G. brebissonii* are the structural characteristics insufficient to distinguish among the resolved clades.

In the case of the Gonatozygaceae, it is clear that there has been an underestimate of the phyletic and probably species diversity. It is not clear why there is so little structural difference between distantly related species. Perhaps the shape is highly constrained by genetic predisposition or, perhaps, there are environmental or physiological constraints on the cell shape. If other structurally simple organisms are as phyletically diverse as the Gonatozygaceae, there may be many more species than are currently recognized.

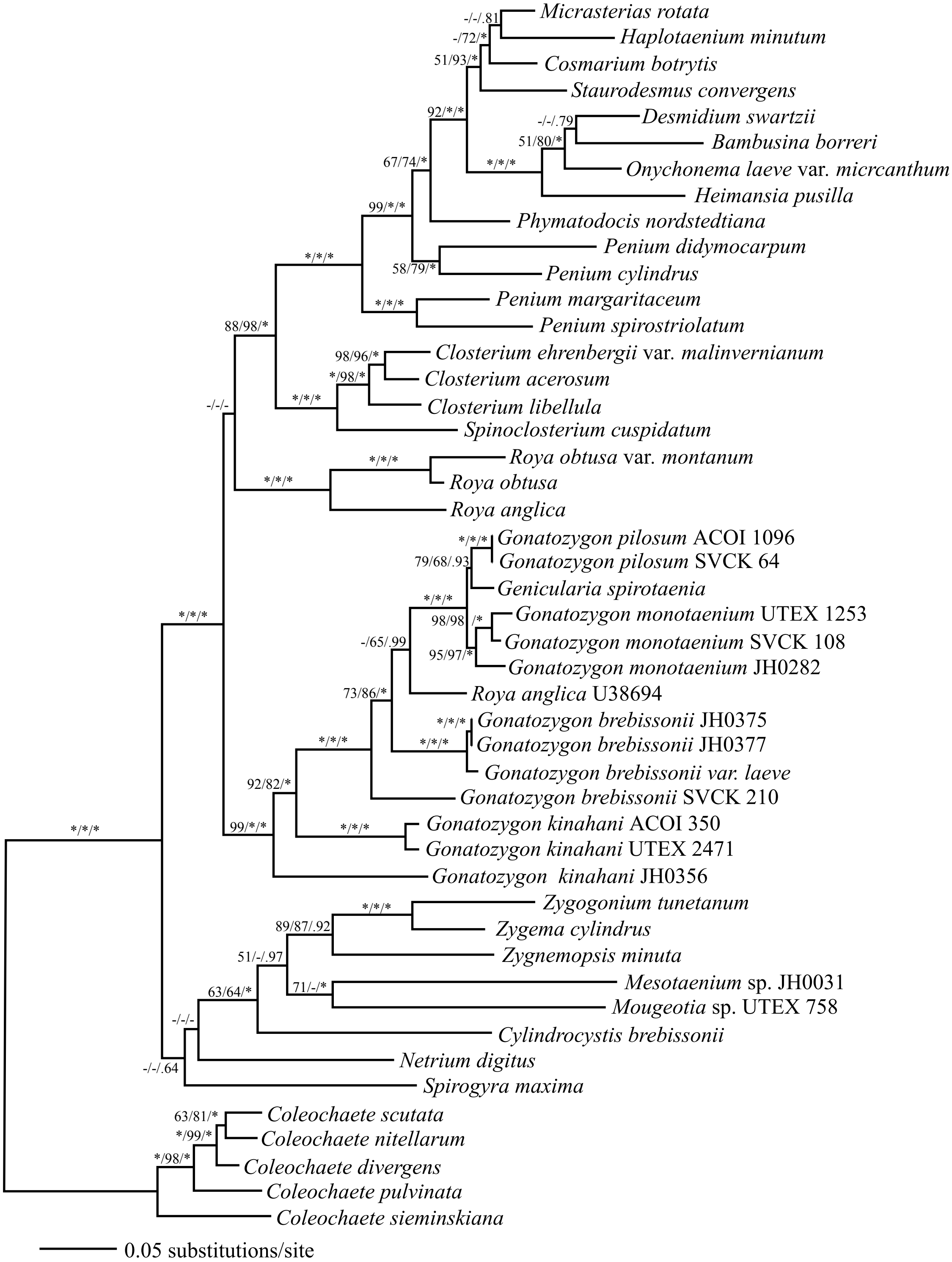


Figure 6.1. Inferred phylogeny of Gonatozygaceae. Maximum likelihood tree from analysis of the genes *rbcL, psaA* and *cox*III. Numbers above branches are MP and ML BS and Bayesian PP, respectively. Values of 100 BS and 1.0 PP are indicated with and asterisk (*). Values less than 50 BS and 0.50 PP are indicated with a dash (-).

Figure 6.2. Light micrographs showing habit of *Gonatozygon* and *Roya*. A. *Gonatozygon monotaenium*; B. *G. brebissonii* var. *laeve*; C. *G. brebissonii*; D. *G. kinahanii* var. *majus*; E. *G. aculeatum*; F. *Roya obtusa* var. *montanum.* Scale bars are 25 micrometers long.

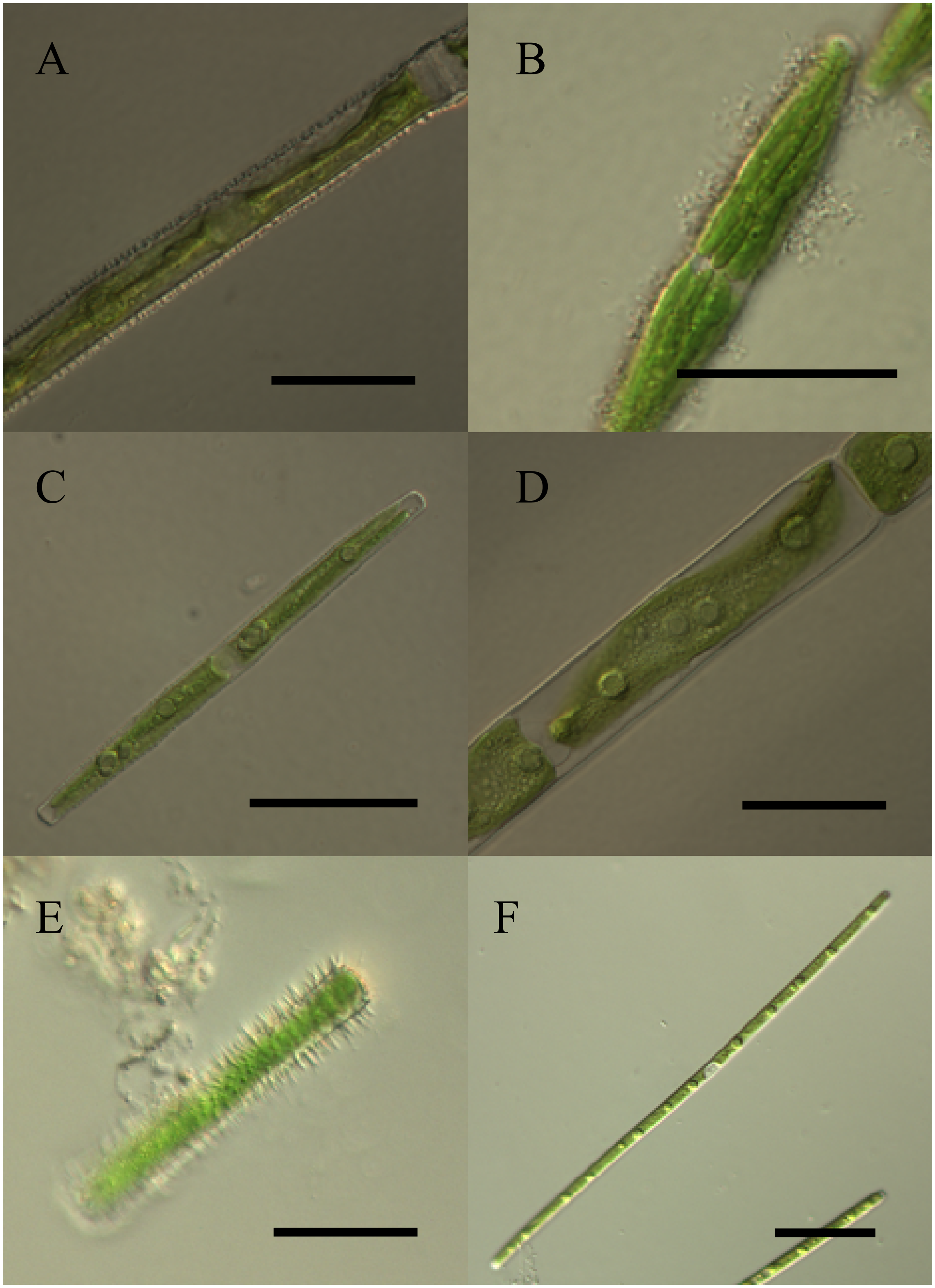

A
B
C
D
E
F

Figure 6.3. Light micrographs showing cell walls of *Gonatozygon* and *Roya*. Cells treated with hypochlorite to show surface ornamentation. A. *G. monotaenium*; B. *G. pilosum*; C. *G. brebissonii*; D. *G. kinahanii* var. *majus*; E. *G. brebissonii* var. *laeve*; F. *G. brebissonii* var. *laeve*; G. *Roya anglica.* Scale bars are 25 micrometers long.

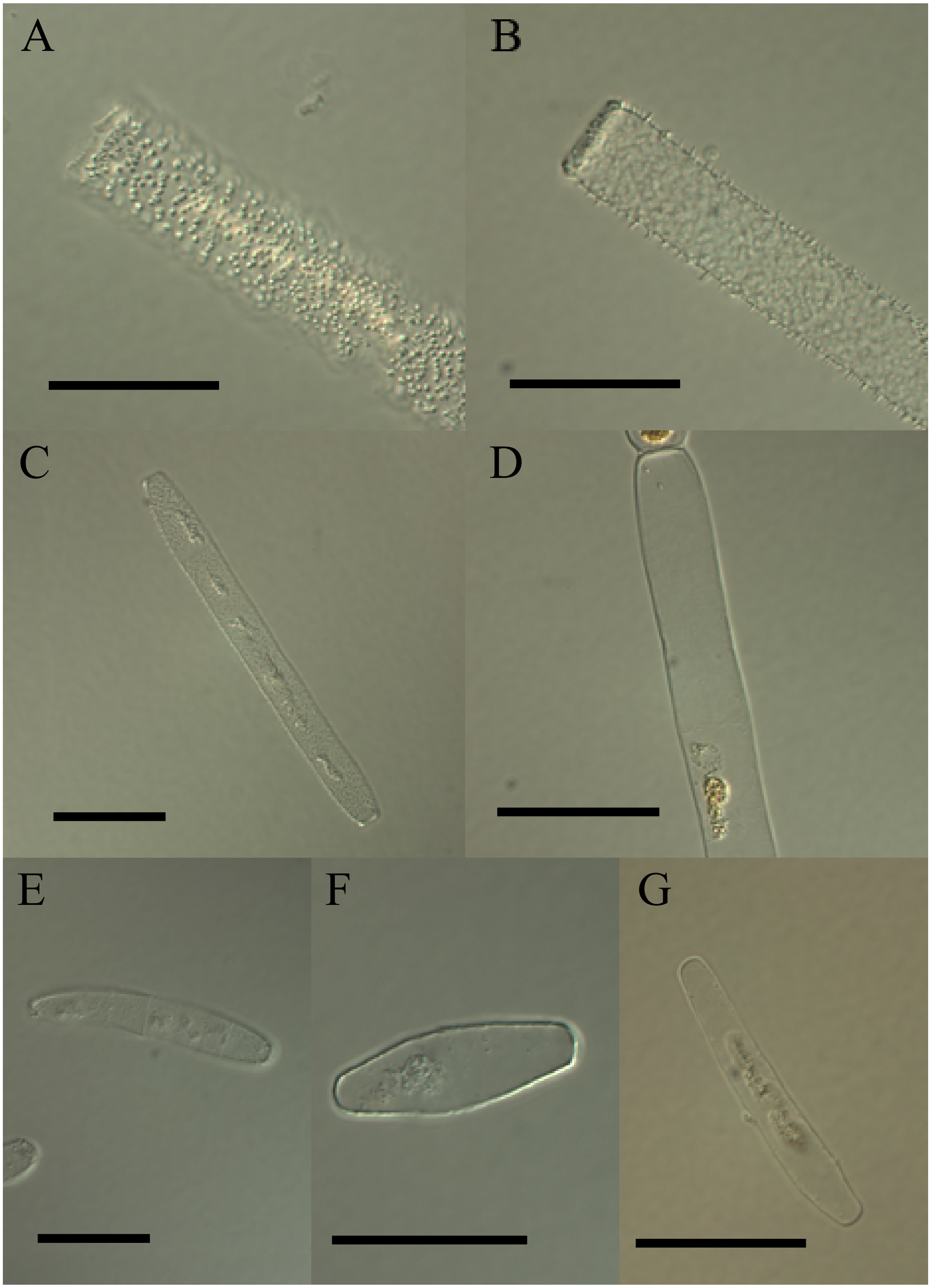

A
B
C
D
E
F
G

Figure 6.4. Comparison of width of *Gonatozygon kinahanii* strains from about 100 randomly sampled cells.

Figure 6.5. Comparison of width of *Gonatozygon brebissonii* strains from about 100 randomly sampled cells.

Figure 6.6 Comparison of length of *Gonatozygon kinahanii* strains from about 100 randomly sampled cells.

Figure 6.7. Comparison of length of *Gonatozygon brebissonii* strains from about 100 randomly sampled cells.

Figure 6.4

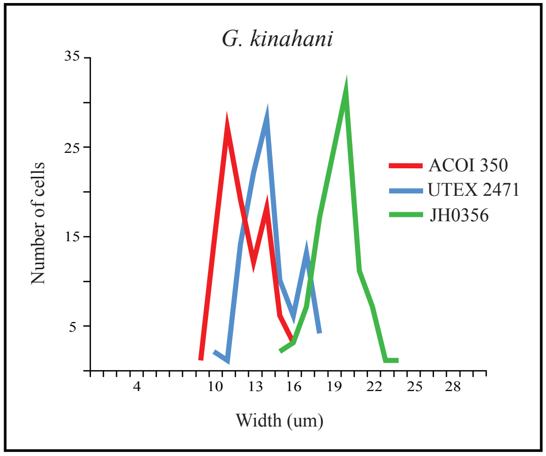


Figure 6.5

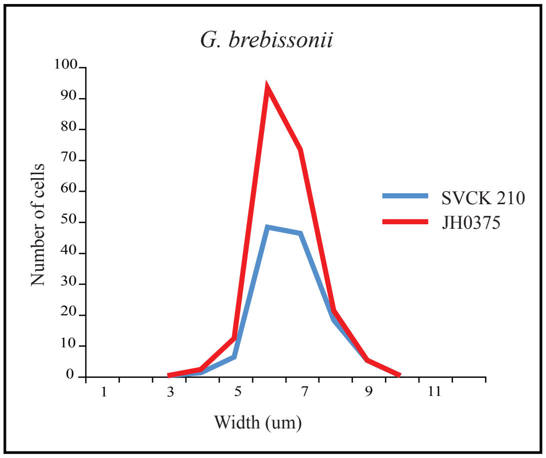


Figure 6.6

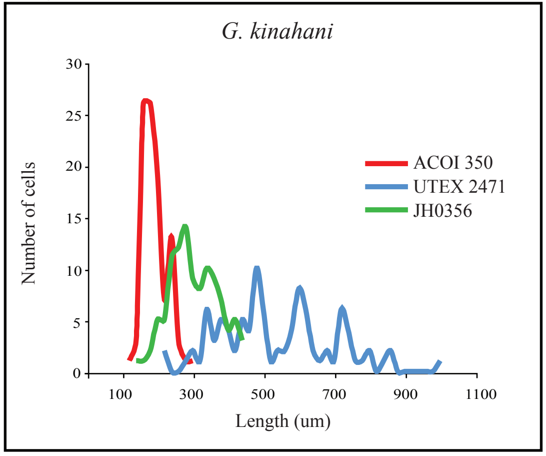
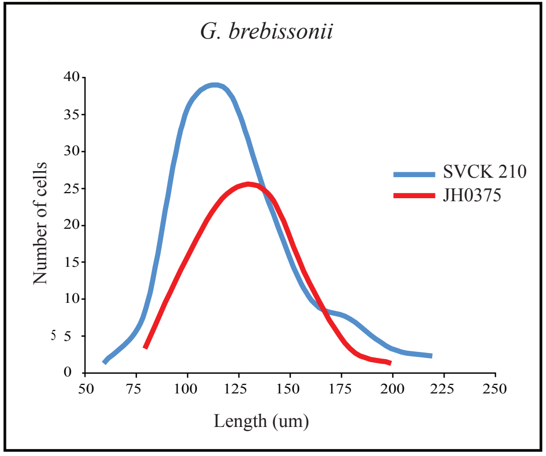


Figure 6.7



Chapter 7. Investigation of the evolutionary history of *Triploceras gracile* (Desmidiaceae).

Abstract

Shifts in cellular organization are thought to be important events in the evolution of complex forms. Among conjugating green algae, shifts from radial to bilateral symmetry are thought to be few and organisms are often assigned to genera based, in part, on the degree of symmetry they display. *Triploceras gracile* is unusual because the middle portions of the cell wall display radial symmetry while the apex is bilaterally symmetric. Previous analysis of the rSSU suggested that *Triploceras* was closely related to *Pleurotaenium*, which is bilaterally symmetric. Analysis of chloroplast and mitochondrial genes indicated that *Triploceras* was actually closely related to the bilaterally symmetric *Micrasterias*. To resolve the apparent contradiction in phylogenetic placement, we sequenced the nuclear genes EF1 α, EIF4 and TUA and analyzed the fragments using phylogenetic methods. We found no strongly supported contradictions between phylogenies from these nuclear markers and the chloroplast and mitochondrial markers suggesting that *Triploceras* is closely related to *Micrasterias* and there has been no confounding evolutionary events such as hybrid speciation, horizontal gene transfer or lineage sorting. We propose that *Triploceras* is actually a highly modified bilaterally symmetric organism closely related to *Micrasterias*.

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Introduction

Triploceras is a unicellular member of a relatively derived lineage of conjugating green algae, the Desmidiaceae (Gontcharov et al., 2003) (Chapter 3). This species is elongate with a well-defined constriction at the isthmus, whorls of spinous processes and a spinous apical lobe (Bailey, 1851). It is unlike most other desmids in that the isthmal part of the cell is radially symmetric in apical view while the apex is bilaterally symmetric (Figure 7.1). There are no obvious affinities between this genus and any other desmid genus although many authors have allied it with other genera that are elongate and have an apical lobe, such as *Triplastrum*, *Ichthyodontum* and *Ichthyocercus* (Gauthier-Lievre, 1960; Prescott, Croasdale, and Vinyard, 1975; Palamar-Mordvintseva, 2003).

The conjugating green algae (Zygnematophyceae) are closely related to land plants (Mattox and Stewart, 1984; McCourt et al., 2000; Karol et al., 2001; Turmel et al., 2006; Hall and Delwiche, 2007)(Chapter 2). One recent phylogenetic analysis based on 76 chloroplast gene sequences from nine charophytes (including land plants) suggested that the conjugating green algae are sister to land plants (Turmel et al., 2006). The conjugating green algae are the most species-rich lineage of charophyte green algae (Chapter 2) and evolution within the group provides direct insight into the evolution of the lineage that gave rise to extant charophytes and land plants.

One major transition in the evolution of land plants was from unicellular to multicellular organization or the thallus (Graham et al., 2000). The increase in organismal complexity was probably preceded by a number of changes in cellular organization including an increase in asymmetry of the cell. In animals, the transition from radial to bilateral symmetry preceded a great radiation of form.

The conjugating green algae is one lineage that contains organisms that are both radially and bilaterally symmetric. Among the conjugating green algae, transitions in symmetry are thought to be rare and species with radial and bilateral symmetry are treated as separate genera (Brook, 1981). In this lineage, bilaterally symmetric organisms are thought to have evolved from radially symmetric ancestors (Teiling, 1952; Prescott et al., 1972; Palamar-Mordvintseva, 2003). However, many genera, including *Triploceras*, were not placed in a phylogenetic context in these studies. It is not known, therefore how many transitions between radial and bilateral symmetry there have been. It is also not clear if *Triploceras* should be considered radially or bilaterally symmetric.

Previous phylogenetic analyses indicated that *Triploceras* was either closely related to *Pleurotaenium* (Gontcharov et al., 2003) (elongate radially symmetric species without either apical processes or whorls of processes) or *Micrasterias* (Chapter 3). *Micrasterias* species are circular or subcircular in lateral view and dorsi-ventrally compressed in apical view. This fundamental dichotomy of growth form, radially symmetric versus bilaterally symmetric, would suggest that the organisms are not closely related. The strong support for a relationship between these two genera based on chloroplast and mitochondrial gene data (Chapter 3) contrasts sharply with that of the SSU topology (Gontcharov et al., 2003) and indicates that there may be a more complex evolutionary history of the genus *Triploceras* than was previously suspected. Potentially confounding events include lineage sorting, horizontal gene transfer and hybrid speciation.

There are no definitive reports of inter-generic hybridization in the conjugating green algae. A number of reports of interspecific hybridizations exist including evidence for species complexes in *Closterium* (Denboh et al., 2003b) and *Pleurotaenium* (Ling and Tyler, 1974). In the case of *Pleurotaenium*, however, the resulting zygotes were not demonstrated to be viable. Additionally, there are a number of reports of interspecific hybridization reported from the wild including many in the genus *Spirogyra* (Transeau, 1951). There have been no reports of horizontal gene transfer or lineage sorting in the conjugating green algae.

To investigate the evolutionary history of the enigmatic Triploceras gracile, we sequenced three nuclear encoded genes from *Triploceras*, *Micrasterias*, *Pleurotaenium* and other related Desmidiaceae to determine the phylogenetic relationships. Our goal was to determine if the relationship between *Triploceras* and *Micrasterias* represents a transition in symmetry and if there has been a confounding event in the evolutionary history of *Triploceras*.

Materials and methods

Unialgal strains were requested from public culture collections or isolated from the wild (Table 7.1). These strains were maintained in Guillards Woods Hole Medium (Nichols, 1973). DNA was extracted using the Phytopure DNA Extraction kit (Amersham, San Francisco, CA) following the manufacturer's protocol with a second chloroform extraction.

The small subunit (SSU) of the nuclear ribosomal DNA (rDNA) was amplified and sequenced using previously published primers and protocols (Marin et al., 1998;

Gontcharov et al., 2003). Portions of the nuclear encoded eukaryotic translation elongation factor 1 alpha (hereafter EF1 α) were amplified from genomic DNA using the nested primers and protocol indicated in Table 7.2. All PCR was performed using 35 cycles of a denaturation at 95º C for 30 seconds, variable annealing temperature for 30 seconds followed by a variable extension time (Table 7.2) at 72º C using Taq polymerase. EF1 α and eukaryotic initiation factor 4 (EIF4) primers were designed by the author. Primers for the amplification of alpha tubulin (TUA) were taken from Kim et al. (2006).

Attempts to amplify EIF4, TUA and other nuclear gene fragments from whole DNA extractions were partially or entirely unsuccessful. In order to obtain these data, RNA was extracted from actively growing cultures using the RNAqueus mini kit (Ambion, Austin, TX) following the manufacturer's instructions. cDNA was synthesized using the Accuscript Kit (Stratagene, La Jolla, CA) following the manufacturer's protocol with oligo dT primers. One microliter of the cDNA was used as a template in subsequent PCR of the EIF4 and TUA using the primers and protocol indicated in Table 2.

Even using cDNA as a template, multiple bands were often encountered, particularly when amplifying TUA, and the fragments were ligated into the pGem T-easy vector (Promega, Madison, WI). Following PCR, products were precipitated using 20% polyethylene glycol (PEG), rinsed in 70% ethanol and resuspended in picopure water. These products were ligated into the pGem vector at 4º C over night (16 hours) and were then cloned into 15 µL of competent *E. coli*. SOC broth was added to 1 milliliter. These bacteria were grown at 37º C for two hours in a shaking water bath, then 200 µL of SOC with bacteria were plated onto 2% agar LB plates with ampicillin. These were allowed to air dry for ten minutes and placed upside down in a growth chamber at 37º C overnight

(16 hours). Between eight and forty-eight of the resulting bacterial colonies were picked and the inserted fragments were amplified off the vector using the primers in Table 2, and an extension time corresponding to the cloned gene fragment. Multiple clones were sequenced and included in analyses.

Table 7.1 Strains investigated and Genbank numbers

Taxon	Strain	GenBank
Closterium peracerosum-strigosum-littorale	NIES67, 68	
Cosmarium botrytis	UTEX 301	
Cosmarium verrucosum	JH0068	
Euastrum crassum var. michiganense	JH0018	
Micrasterias depauperata	JH0364	
Micrasterias foliaceae	NIES 297	
Micrasterias laticeps	JH0239	
Micrasterias muricata	JH0119	
Micrasterias muricata	JH0120	
Micrasterias muricata	JH0179	
Micrasterias pinnatafida	JH0106	
Micrasterias radiata	JH0064	
Micrasterias radiata	JH0226	
Micrasterias rotata	UTEX 1941	
Micrasterias truncata	JH0017	
Phymatodocis nordstedtiana	SAG 47.89	
Pleurotaenium baculoides	JH0008	
Pleurotaenium constrictum	JH0135	
Triploceras gracile	JH0215	
Triploceras gracile	SAG 24.82	
Xanthidium hastiferum	JH0054	
Actinotaenium cucurbita		AY964132.1
Cosmarium costatum		AY964126.1
Cosmarium norimbergense var. depressum		AY964138.1
Cosmarium obsoletum		AY964128.1
Cosmarium pseudoconnatum		AY964150.1
Cosmarium tenue		AY964127.1
Desmidium swartzii		AJ428133.1
Euastrum oblongum		AJ428095.1
Euastrum pectinatum		AJ549227.1
Euastrum pinnatum		AJ428096.1
Groenbladia neglecta		AJ428119.1
Haplotaenium minutum		AJ428090.1
Heimansia pusilla		AJ428125.1
Hyalotheca dissiliens		AJ428120.1
Micrasterias crux-melitensis		AJ428097.1
Micrasterias fimbriata		AJ428098.1
Onychonema laeve		AJ428127.1
Phymatodocis nordstedtiana		AJ428122.1
Pleurotaenium ehrenbergii		AJ428132.1
Pleurotaenium trabecula		AJ428131.1
Staurastrum cristatum		AJ428110.1
Staurastrum ophiura		AJ428104.1
Staurastrum pingue		AJ428109.1
Staurodesmus bulnheimii		AJ428111.1
Staurodesmus convergens		AY964143.1
Staurodesmus mucronatus		AJ428103.1
Tetmemorus brebissonii		AJ428091.1

Triploceras gracile	AJ428089.1
Xanthidium antilopaeum	AJ428093.1
Xanthidium armatum	AJ428094.1
Xanthidium brebissonii	AJ428092.1

Table 7.2 PCR primers and conditions

Gene	Primer	Temp.	Time
18S	18S A - CTGGTTGATYCTGCCAGT	52 C	45 sec
	18S B - CYGCAGGTTCACCTACRG		
EF1 α	**EF1F1 - GCTGAGATGAACAAGAGG**	52 C	45 sec
	EF1R2 - GCCGACCTTCTTCAGGTAGG		
EF1 α nested	**F6 Mic - CACCATTTCCTCATAGCGGG**	52 C	45 sec
	R4 Mic - GCCTGGGTGCTGGACAAGCTCAAGG		
EIF4	**EIF4F - CGCCGCAAGGTGGACTGG**	52 C	45 sec
	EIF4R - GTCCTGCAGCATGCGCTCGTC		
TUA	TUAF22/23 - CACATCGGNCARGCCGGNRTCCA	48 C	120 sec
	TUAR 1268/26 - GCYTCRGARAAYTCNCCYTCCTCCAT		
TUA nested	TUAF58/25 - TGCTGGGAGCTNTACTGCCTNGAGCA	48 C	120 sec
	TUAR 1248/26 - TCCTCCATNCCYTCNCCNACRTACCA		
pGEM vector	**T7 F - TAATACGACTCACTATAGGG**	52 C	variable
	M13 R - ACCATGATTACGCCAAG		

Notes: Primers in bold were designed by the author. Temp. refers to the annealing temperature of the PC reaction. Time refers to the length of the extension time in seconds. When amplifying from vector, the extension time depended on the length of the target fragment.

Gene fragments were aligned by eye in MacClade 4.0 (Maddison and Maddison, 2000). Introns, indels, and unalignable portions were excluded from the analysis (positions 1-211, 368-589, 629-676, 776-961, 986-1127 of EF1 α; positions 181-336 of EIF4; and positions 85-87, 289-381, 588-678, 1315-1365 of TUA). Either *Phymatodocis* or *Closterium* were used to root the trees. Phylogenetic analyses were performed in Paup* v. 4 b.10 (Swofford, 2003) and MrBayes v. 3.1 (Huelsenbeck and Ronquist, 2000). Other taxa belonging to other lineages separating *Micrasterias* and *Triploceras* in the SSU phylogeny were also included in the analysis. Under the Parsimony criterion (MP), the best tree was searched for heuristically with starting trees constructed by one hundred random taxon addition sequences and using tree bisection-reconnection (TBR) branch swapping. Support for taxon bipartitions was estimated from 500 bootstrap pseudoreplicates with ten random taxon addition sequences and TBR swapping. For likelihood analyses, the models and parameter settings were selected based on the output of Mr. Modeltest v.2 (Nylander, 2004): SSU, GTR+I+G; EF1, SYM+G; EIF4, GTR+G; TUA, GTR+I+G. Under the likelihood criterion (ML), the best tree was calculated from ten random addition sequence replicates and TBR branch swapping. Support was estimated from 100 bootstrap replicates with three random taxon addition sequences and TBR swapping. Under Bayesian Inference (BI), tree space was explored using a markov chain monte carlo method with four chains run for 4,000,000 generations sampled every 100 generations. The first 2501 trees, well into the stationary phase in all datasets, were discarded as burnin.

Results

Sequences from GenBank were concatenated into a dataset with only Desmidiales, mostly Desmidiaceae and the alignment was analyzed using all previously described methods. In this analysis, the position of *Triploceras* was not resolved (Figure 7.2), but its placement differed from that of the published SSU topology.

Direct sequencing of EF1 α from the nuclear DNA, revealed two slightly different sequences in *Triploceras*. These sequences were identical to or differed by one base pair from those obtained for all *Micrasterias* species (Figure 7.3), resulting in two essentially identical most parsimonious trees (Figure 7.3A, B). Likelihood analyses showed similar results differing only in the relative support for relationships among outgroup taxa (Figure 7.3C, D).

Fragments of EIF4 were obtained by cloning PCR products amplified from either the total nuclear DNA or cDNA prepared from RNA (which, presumably, would lack introns). Parsimony analysis of this dataset found four equally parsimonious trees that were mostly unresolved but showed a sister relationship between *Triploceras* and *Micrasterias* (Figure 7.4B, E) as well as a relationship between *Triploceras* and *Pleurotaenium* (Figure 7.4A) among others (Figure 7.4D). No placement of *Triploceras* was statistically supported in parsimony analysis of EIF4. Under the likelihood criterion, only a relationship between *Triploceras* and *Micrasterias muricata* was discovered, although this relationship was not statistically supported in either ML (>50; Figure 7.4C) or BI (0.54; Figure 7.4F). Multiple accessions for each strain were included to show the variation induced, presumably, from cloning individual PCR products.

Sequences of TUA were not available from *Closterium*, and it was not possible to amplify a fragment from *Phymatodocis* (the two taxa selected as outgroups), so these trees are shown as unrooted cladograms. In parsimony analysis, sixty-three most parsimonious trees were encountered. These differed only in the placement of the terminal taxa. Figure 7.5A shows one of these trees with proportionate branch lengths, while 7.5B shows the strict consensus of the sixty-three most parsimonious trees (branch lengths not significant). All trees showed a sister relationship between *Micrasterias muricata* and *Triploceras* as well as a monophyletic *Micrasterias* that included *Triploceras* (Figure 7.5A, B). In the ML analysis, the internal nodes were not resolved and *Triploceras* appeared sister to *Micrasterias foliacea* in the most likely tree, but *Micrasterias* was not monophyletic (Figure 7.5C). Under Bayesian Inference, a monophyletic *Micrasterias* was present in the consensus tree (Figure 7.5D), and *Triploceras* appeared sister to *Micrasterias foliacea* with moderate support (0.89).

Discussion

The discovered relationship between *Micrasterias* and *Triploceras* was not expected. As previously noted, these organisms are very different structurally and previous phylogenetic analysis of ribosomal SSU found no relationship between these genera (Gontcharov et al., 2003). Attempts to reproduce those experiments resulted in a topology that was consistent with all other sampled markers (Figure 7.2) but inconsistent with the published SSU topology (Gontcharov et al., 2003). It is likely that the placement of *Triploceras* discovered by Gontcharov et al. (2003) was the result of either a spurious sequence that was assigned to *Triploceras*, a peculiarity of their alignment, or taxon sampling. Gontcharov et al. (2003) did not include either of the species of *Micrasterias* that most often appeared sister to *Triploceras*: *Micrasterias foliaceae* and *M. muricata*.

If taken at face value, the results presented by Gontcharov et al. (2003) may indicate that there was some more complex evolutionary history that gave rise to the different gene trees. These include, but are not limited to, horizontal gene transfer, lineage sorting and hybridization (Linder and Rieseberg, 2004). It is possible to distinguish among these potentially confounding factors and refute the hypothesis that one of those mechanisms is responsible for the apparent incongruence.

In all three cases, a past event will result in a gene tree that is not a reflection of the species tree (Figure 7.6). In horizontal gene transfer, a gene or portion of a genome is transferred from one organism to another - potentially distantly related - organism. Viruses and bacteria are often suspected as vector agents, but other biological processes may be involved. When estimating phylogenies based on a single gene with a history of horizontal transfer, one would find an unexpected relationship between two organisms (Figure 7.6A). In general, horizontal gene transfers can be detected by investigating multiple loci and increasing taxon sampling to determine where the horizontally transferred sequence originated.

Lineage sorting can be more difficult to detect. In this case, the relationships of extant taxa are distorted by past sorting events. For example, if a lineage of organisms contained multiple alleles of a particular gene at some point in the past, but these alleles were lost over time, then the apparent relationship of extant taxa based on that gene would not be indicative of the species relationships, but rather - if that gene tree could be mapped onto a phylogenetic tree showing the true evolutionary history - it would indicate the history of allele or gene evolution and extinctions (Figure 7.6B). Again, lineage sorting can often be detected by sampling multiple genes with independent evolutionary histories.

Hybrid speciation is very different from the two preceding processes. In its simplest form, hybridization involves the union of whole genomes from two different species to give rise to a new morphologically distinct species. Although rare in animals, this process is comparatively common in plants and is considered an important evolutionary phenomenon (Hegarty and Hiscock, 2005). Hybrid organisms have some peculiar genomic features and the genes in the nuclear genome can have a number of fates. However, when sampled in a phylogenetic context, the nuclear genome will often appear to be a mix of the two parental species (Nishimoto et al., 2003; Linder and Rieseberg, 2004; Hegarty and Hiscock, 2005; Kameyama et al., 2005).

The general congruence among genes sampled in this study suggests that horizontal gene transfer is not responsible for the relationships. It is possible that the ribosomal genes were transferred from another organism, but this analysis of the rSSU indicates a possible relationship between *Triploceras* and *Micrasterias* (Figure 7.2). This general congruence among the molecules also suggests that lineage sorting does not seem to explain the close relationship between *Micrasterias* and *Triploceras*. If lineage sorting were a factor, one would expect to find different relationships when analyzing different genes, which was not the case. The absence of incongruous trees also essentially negates the hypothesis of a past hybridization event. To be certain, one would have to sample all the genes in the genomes of *Micrasterias*, *Triploceras* and some outgroup taxa. At the present, there is no compelling reason to do so.

In the published *rbcL*, *psaA* and *cox*III datasets (Chapter 3), as well as the newly presented EF1 α dataset, *Triploceras* and some *Micrasterias* species shared identical primary sequence (Figure 7.3). Most sampled genes indicated that species of *Micrasterias* and *Triploceras* had little difference in primary sequence of the gene fragments, but there was also little difference between primary sequence of coding regions in *Micrasterias* and outgroup taxa such as *Euastrum* and *Cosmarium* (Figures 7.4, 7.5). This made estimation of the relationships among the species particularly difficult in EIF4 and TUA datasets, as indicated by low bootstrap support and low posterior probabilities (Figures 7.4, 7.5). These genes are probably not sufficiently variable to resolve these relationships, and future studies investigating species-level relationships will likely have to rely on noncoding sequence such as introns or microsatellites. However, in the one instance where intron sequences were available, the EF1 α dataset, the intron sequence *Micrasterias* and other Desmidiaceae were not alignable (data not shown).

The apparent relationship between *Micrasterias* and *Triploceras* may have been previously overlooked because of taxon sampling within *Micrasterias*. In my analyses, *Triploceras* was found at the base of the *Micrasterias* clade, usually associated with *Micraterias foliacea* or *M. muricata*. Neither of these taxa was included in previous SSU studies (Gontcharov et al., 2003). These taxa are unique among *Micrasterias* species for different reasons. *Micrasterias foliacea*, although structurally similar to other highly dissected species of *Micrasterias,* forms long chains of cells held together by their apical processes (Lorch and Engels, 1979). *Micrasterias muricata*, on the other hand, is structurally unlike most other species of *Micrasterias* in that it lacks the compressed lateral lobes so typical of *Micrasterias* and, instead, has long tubular processes similar to many species of *Staurastrum*. The discovered phylogenetic relationships among *Micrasterias* species brings to light aspects of cellular structure that had been previously overlooked.

First, it is interesting that the filamentous *M. foliacea* is among the basal lineages of *Micrasterias*. Its position at the base of the clade suggests that the ancestor to extant *Micrasterias* species may have had many dissected lobes as found in *M. foliacea* and many other *Micrasterias* species. Also, the close relationship between *Micrasterias* and *Triploceras* requires careful consideration of the use of symmetry as a systematic characteristic. *Triploceras* is often considered radially symmetric (Brook, 1981), although its apical lobe is certainly bilaterally symmetric. If it is considered radially symmetric, then we must conclude that transitions from radial to bilateral symmetry (or the reverse) have happened many times in the evolution of desmids and at least once in the *Micrasterias* lineage. If *Triploceras* is bilaterally symmetric, then it would seem that the portion of the cell that appears to be radially symmetric is in fact a highly modified bilaterally symmetric semicell. Accepting the possible ambiguity of radial symmetry, one must also consider the possibility that many other desmid genera which appear to be radially symmetric are, in fact, bilaterally symmetric.

This seems to be the case for a great many "radially" symmetric desmids. Filamentous desmids such as *Didymoprium grevillei*, *Bambusina borreri*, and *Hyalotheca dissiliens*, often have lateral tubercles that indicate that the cell wall is not radial, but rather bilateral. In all cases, it is still possible that the chloroplast (and possibly other cytoplasmic contents) may be arranged in radial symmetry, but the cell wall is not.

Under this assumption, the whorls of processes in *Triploceras* could be interpreted as reduced, multidimensional lobes. The genetic basis for such a transition is unknown, but a potentially interesting line of research that may prove to be important to our understanding or cellular development and morphogenesis in desmids.

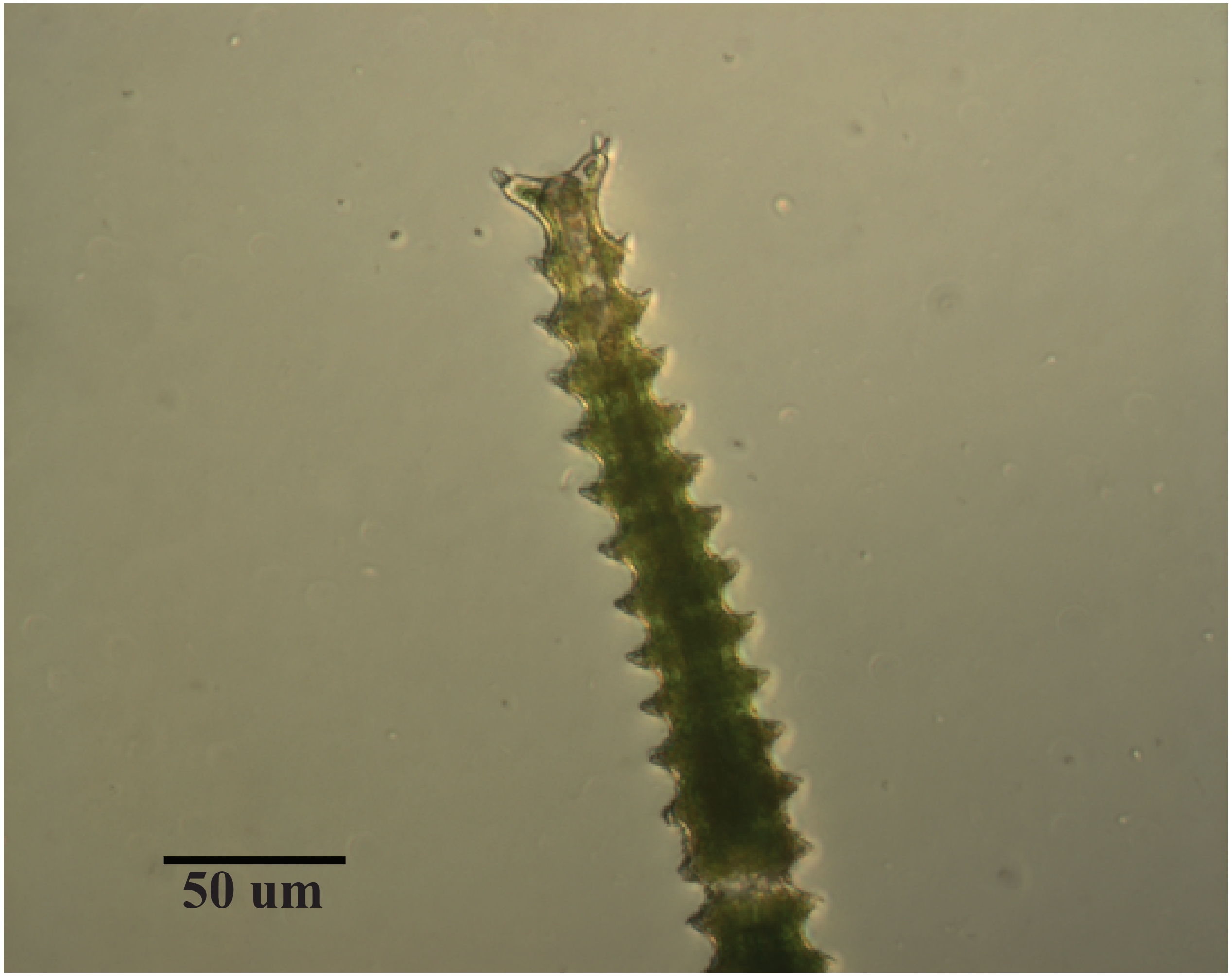


Figure 7.1 Light micrograph of *Triploceras gracile* var. *gracile*

Figure 7.2. Genealogies based on rSSU. A. is one of the 115 most parsimonious trees. Numbers above branches are bootstrap values. A dash indicates support less than 50. B. is the strict consensus of the 115 MP trees. C. Maximum Likelihood tree. F. Likelihood tree from Bayesian Inference. Numbers above branches are posterior probabilities. A dash represents support less than 0.50.

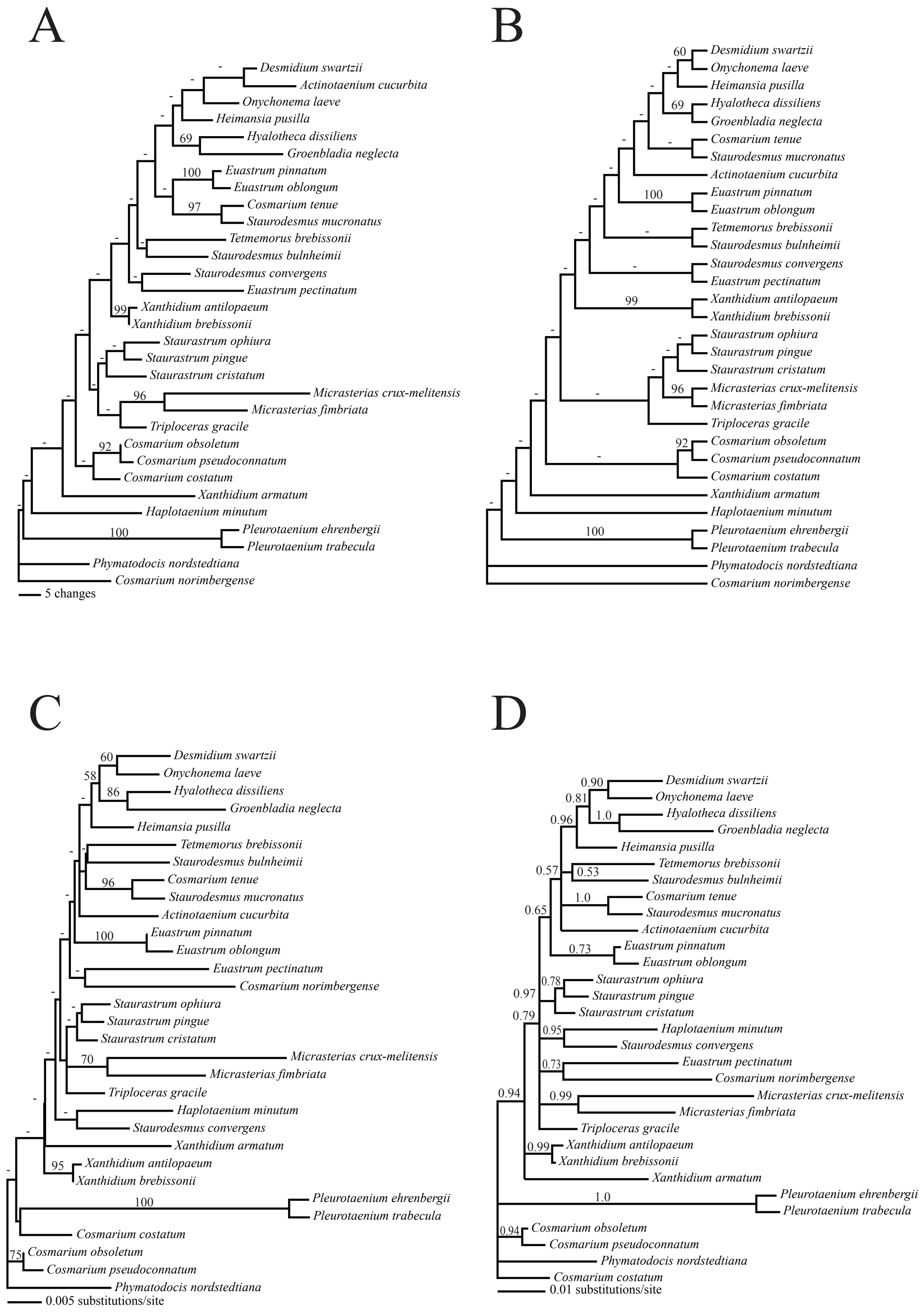
A
Desmidium swartzii
Actinotaenium cucurbita
Onychonema laeve
Heimansia pusilla
69
Hyalotheca dissiliens
Groenbladia neglecta
100
Euastrum pinnatum
Euastrum oblongum
97
Cosmarium tenue
Staurodesmus mucronatus
Tetmemorus brebissonii
Staurodesmus bulnheimii
Staurodesmus convergens
Euastrum pectinatum
99
Xanthidium antilopaeum
Xanthidium brebissonii
Staurastrum ophiura
Staurastrum pingue
Staurastrum cristatum
96
Micrasterias crux-melitensis
Micrasterias fimbriata
Triploceras gracile
92
Cosmarium obsoletum
Cosmarium pseudoconnatum
Cosmarium costatum
Xanthidium armatum
Haplotaenium minutum
100
Pleurotaenium ehrenbergii
Pleurotaenium trabecula
Phymatodocis nordstedtiana
Cosmarium norimbergense
5 changes
B
60
Desmidium swartzii
Onychonema laeve
Heimansia pusilla
69
Hyalotheca dissiliens
Groenbladia neglecta
Cosmarium tenue
Staurodesmus mucronatus
Actinotaenium cucurbita
100
Euastrum pinnatum
Euastrum oblongum
Tetmemorus brebissonii
Staurodesmus bulnheimii
Staurodesmus convergens
Euastrum pectinatum
99
Xanthidium antilopaeum
Xanthidium brebissonii
Staurastrum ophiura
Staurastrum pingue
Staurastrum cristatum
96
Micrasterias crux-melitensis
Micrasterias fimbriata
Triploceras gracile
92
Cosmarium obsoletum
Cosmarium pseudoconnatum
Cosmarium costatum
Xanthidium armatum
Haplotaenium minutum
100
Pleurotaenium ehrenbergii
Pleurotaenium trabecula
Phymatodocis nordstedtiana
Cosmarium norimbergense
C
60
Desmidium swartzii
58
Onychonema laeve
86
Hyalotheca dissiliens
Groenbladia neglecta
Heimansia pusilla
Tetmemorus brebissonii
Staurodesmus bulnheimii
96
Cosmarium tenue
Staurodesmus mucronatus
Actinotaenium cucurbita
100
Euastrum pinnatum
Euastrum oblongum
Euastrum pectinatum
Cosmarium norimbergense
Staurastrum ophiura
Staurastrum pingue
Staurastrum cristatum
70
Micrasterias crux-melitensis
Micrasterias fimbriata
Triploceras gracile
Haplotaenium minutum
Staurodesmus convergens
Xanthidium armatum
95
Xanthidium antilopaeum
Xanthidium brebissonii
100
Pleurotaenium ehrenbergii
Pleurotaenium trabecula
Cosmarium costatum
75
Cosmarium obsoletum
Cosmarium pseudoconnatum
Phymatodocis nordstedtiana
0.005 substitutions/site
D
0.90
Desmidium swartzii
0.81
Onychonema laeve
0.96
1.0
Hyalotheca dissiliens
Groenbladia neglecta
Heimansia pusilla
Tetmemorus brebissonii
0.57
0.53
Staurodesmus bulnheimii
1.0
Cosmarium tenue
0.65
Staurodesmus mucronatus
Actinotaenium cucurbita
0.73
Euastrum pinnatum
Euastrum oblongum
0.78
Staurastrum ophiura
Staurastrum pingue
0.97
Staurastrum cristatum
0.79
0.95
Haplotaenium minutum
Staurodesmus convergens
0.73
Euastrum pectinatum
Cosmarium norimbergense
0.94
0.99
Micrasterias crux-melitensis
Micrasterias fimbriata
Triploceras gracile
0.99
Xanthidium antilopaeum
Xanthidium brebissonii
Xanthidium armatum
1.0
Pleurotaenium ehrenbergii
Pleurotaenium trabecula
0.94
Cosmarium obsoletum
Cosmarium pseudoconnatum
Phymatodocis nordstedtiana
Cosmarium costatum
0.01 substitutions/site

Figure 7.3. Genealogies based on EF1. A, B are the two most parsimonious trees. Numbers above branches are bootstrap values. A dash indicates support less than 50. C. Maximum Likelihood tree. D. Likelihood tree from Bayesian Inference. Numbers above branches are posterior probabilities. A dash represents support less than 0.50.

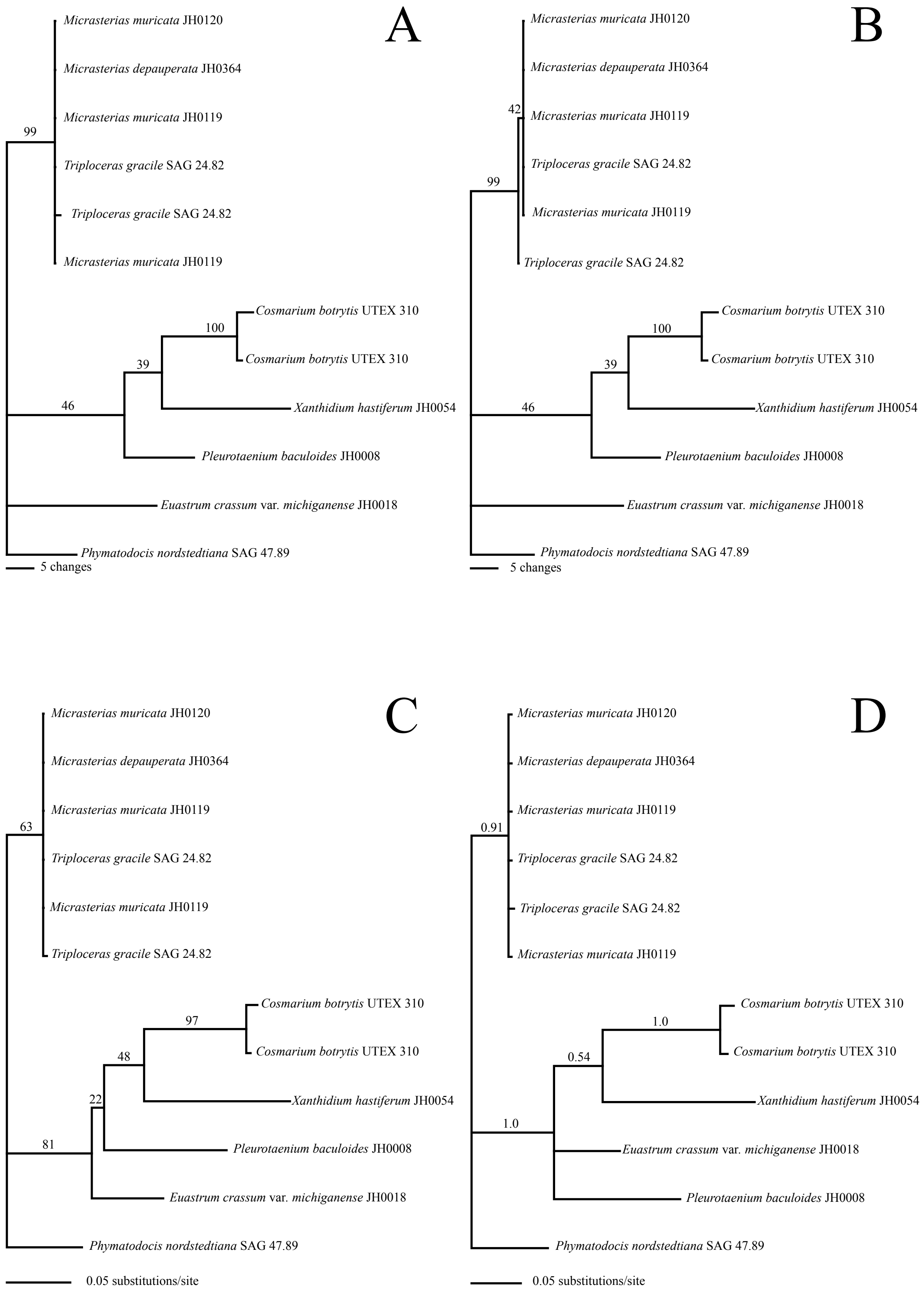

A
Micrasterias muricata JH0120
Micrasterias depauperata JH0364
Micrasterias muricata JH0119
Triploceras gracile SAG 24.82
Triploceras gracile SAG 24.82
Micrasterias muricata JH0119
99
Cosmarium botrytis UTEX 310
Cosmarium botrytis UTEX 310
100
39
Xanthidium hastiferum JH0054
46
Pleurotaenium baculoides JH0008
Euastrum crassum var. michiganense JH0018
Phymatodocis nordstedtiana SAG 47.89
5 changes
B
Micrasterias muricata JH0120
Micrasterias depauperata JH0364
42
Micrasterias muricata JH0119
Triploceras gracile SAG 24.82
99
Micrasterias muricata JH0119
Triploceras gracile SAG 24.82
Cosmarium botrytis UTEX 310
100
Cosmarium botrytis UTEX 310
39
Xanthidium hastiferum JH0054
46
Pleurotaenium baculoides JH0008
Euastrum crassum var. michiganense JH0018
Phymatodocis nordstedtiana SAG 47.89
5 changes
C
Micrasterias muricata JH0120
Micrasterias depauperata JH0364
Micrasterias muricata JH0119
63
Triploceras gracile SAG 24.82
Micrasterias muricata JH0119
Triploceras gracile SAG 24.82
Cosmarium botrytis UTEX 310
97
Cosmarium botrytis UTEX 310
48
22
Xanthidium hastiferum JH0054
81
Pleurotaenium baculoides JH0008
Euastrum crassum var. michiganense JH0018
Phymatodocis nordstedtiana SAG 47.89
0.05 substitutions/site
D
Micrasterias muricata JH0120
Micrasterias depauperata JH0364
Micrasterias muricata JH0119
0.91
Triploceras gracile SAG 24.82
Triploceras gracile SAG 24.82
Micrasterias muricata JH0119
Cosmarium botrytis UTEX 310
1.0
Cosmarium botrytis UTEX 310
0.54
Xanthidium hastiferum JH0054
1.0
Euastrum crassum var. michiganense JH0018
Pleurotaenium baculoides JH0008
Phymatodocis nordstedtiana SAG 47.89
0.05 substitutions/site

Figure 7.4. Genealogies based on EIF4. A, B, D, and E are the four most parsimonious trees. Numbers above branches are bootstrap values. A dash indicates support less than 50. C. Maximum Likelihood tree. F. Likelihood tree from Bayesian Inference. Numbers above branches are posterior probabilities. A dash represents support less than 0.50.

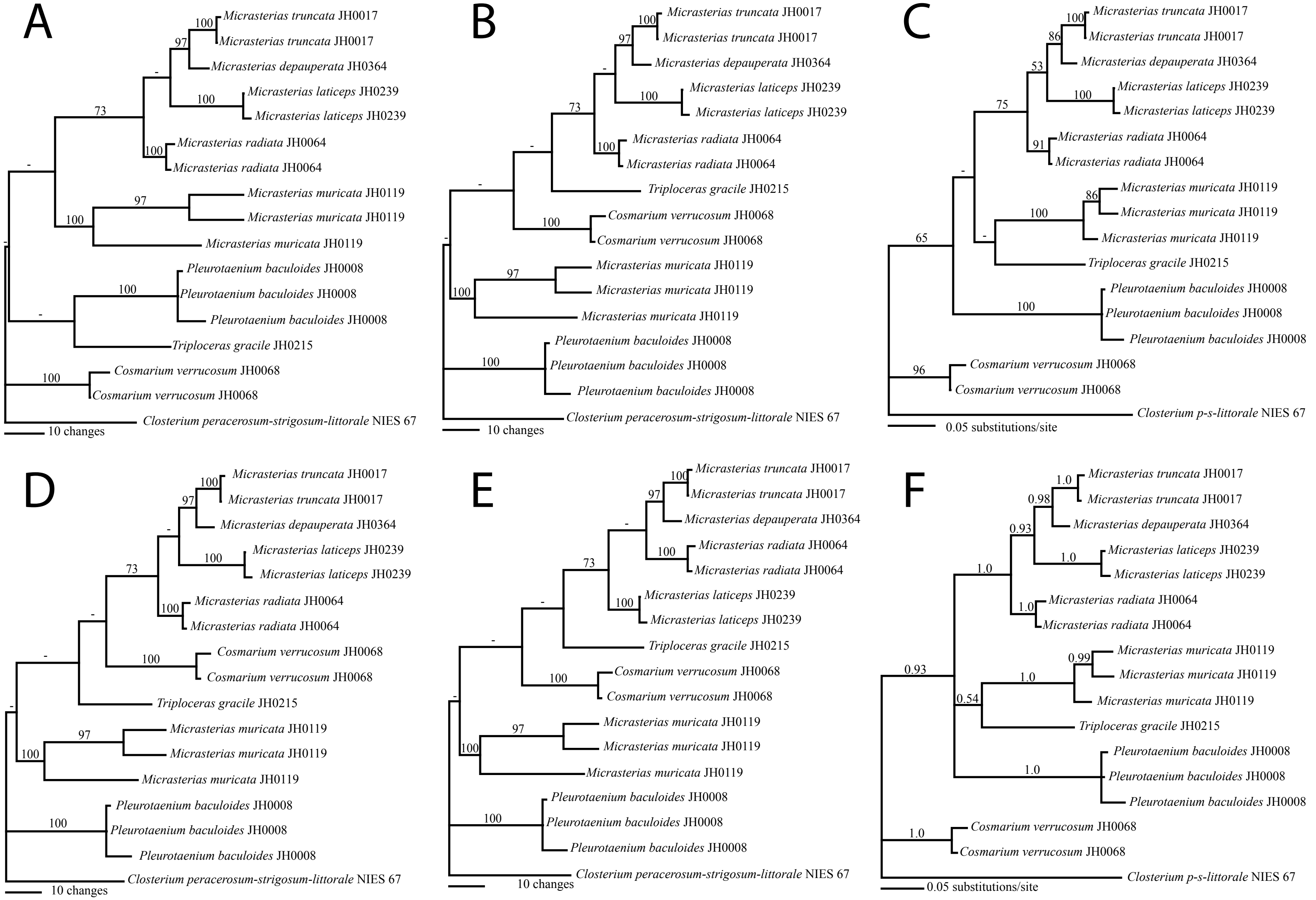

A
Micrasterias truncata JH0017
Micrasterias truncata JH0017
Micrasterias depauperata JH0364
Micrasterias laticeps JH0239
Micrasterias laticeps JH0239
Micrasterias radiata JH0064
Micrasterias radiata JH0064
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Triploceras gracile JH0215
Cosmarium verrucosum JH0068
Cosmarium verrucosum JH0068
Closterium peracerosum-strigosum-littorale NIES 67
100
97
-
100
73
100
-
97
100
100
-
-
100
10 changes
B
Micrasterias truncata JH0017
Micrasterias truncata JH0017
Micrasterias depauperata JH0364
Micrasterias laticeps JH0239
Micrasterias laticeps JH0239
Micrasterias radiata JH0064
Micrasterias radiata JH0064
Triploceras gracile JH0215
Cosmarium verrucosum JH0068
Cosmarium verrucosum JH0068
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Closterium peracerosum-strigosum-littorale NIES 67
100
97
-
100
73
100
-
-
100
97
100
-
100
10 changes
C
Micrasterias truncata JH0017
Micrasterias truncata JH0017
Micrasterias depauperata JH0364
Micrasterias laticeps JH0239
Micrasterias laticeps JH0239
Micrasterias radiata JH0064
Micrasterias radiata JH0064
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Triploceras gracile JH0215
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Cosmarium verrucosum JH0068
Cosmarium verrucosum JH0068
Closterium p-s-littorale NIES 67
100
86
53
100
75
91
86
100
-
-
65
100
96
0.05 substitutions/site
D
Micrasterias truncata JH0017
Micrasterias truncata JH0017
Micrasterias depauperata JH0364
Micrasterias laticeps JH0239
Micrasterias laticeps JH0239
Micrasterias radiata JH0064
Micrasterias radiata JH0064
Cosmarium verrucosum JH0068
Cosmarium verrucosum JH0068
Triploceras gracile JH0215
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Closterium peracerosum-strigosum-littorale NIES 67
100
97
-
100
73
100
-
100
-
-
97
100
100
10 changes
E
Micrasterias truncata JH0017
Micrasterias truncata JH0017
Micrasterias depauperata JH0364
Micrasterias radiata JH0064
Micrasterias radiata JH0064
Micrasterias laticeps JH0239
Micrasterias laticeps JH0239
Triploceras gracile JH0215
Cosmarium verrucosum JH0068
Cosmarium verrucosum JH0068
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Closterium peracerosum-strigosum-littorale NIES 67
100
97
-
100
73
100
-
-
100
-
97
100
100
10 changes
F
Micrasterias truncata JH0017
Micrasterias truncata JH0017
Micrasterias depauperata JH0364
Micrasterias laticeps JH0239
Micrasterias laticeps JH0239
Micrasterias radiata JH0064
Micrasterias radiata JH0064
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Micrasterias muricata JH0119
Triploceras gracile JH0215
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Pleurotaenium baculoides JH0008
Cosmarium verrucosum JH0068
Cosmarium verrucosum JH0068
Closterium p-s-littorale NIES 67
1.0
0.98
0.93
1.0
1.0
1.0
0.99
1.0
0.93
0.54
1.0
1.0
0.05 substitutions/site

Figure 7.5. Genealogies based on TUA. A. One of 63 most parsimonious trees showing branchlengths. Numbers above branches are bootstrap values. A dash represents support less than 50. B. Strict consensus of 63 most parsimonious trees. C. Maximum Likelihood tree. D. Likelihood tree from Bayesian Inference. Numbers above branches are posterior probabilities. A dash represents support less than 0.50.

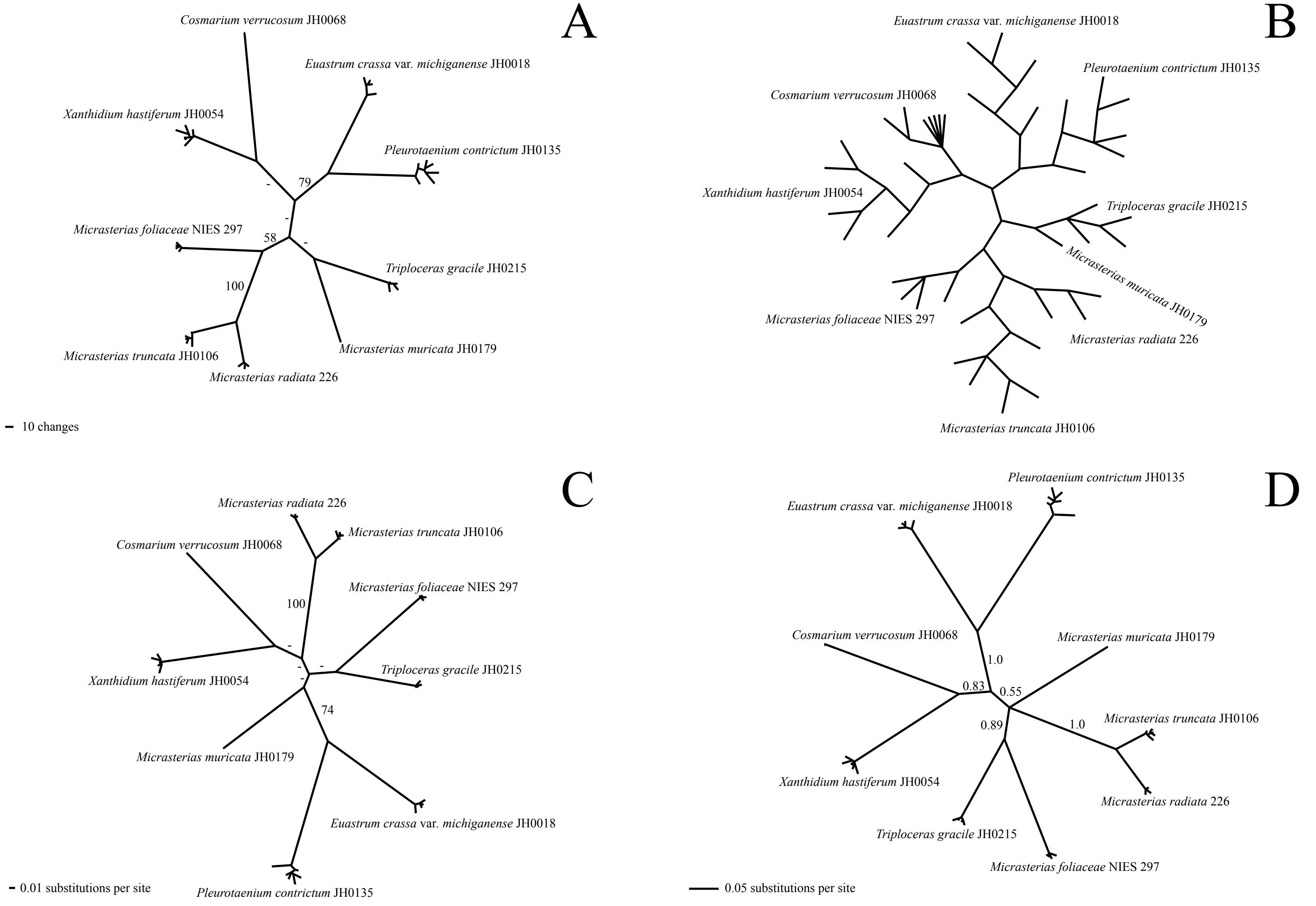
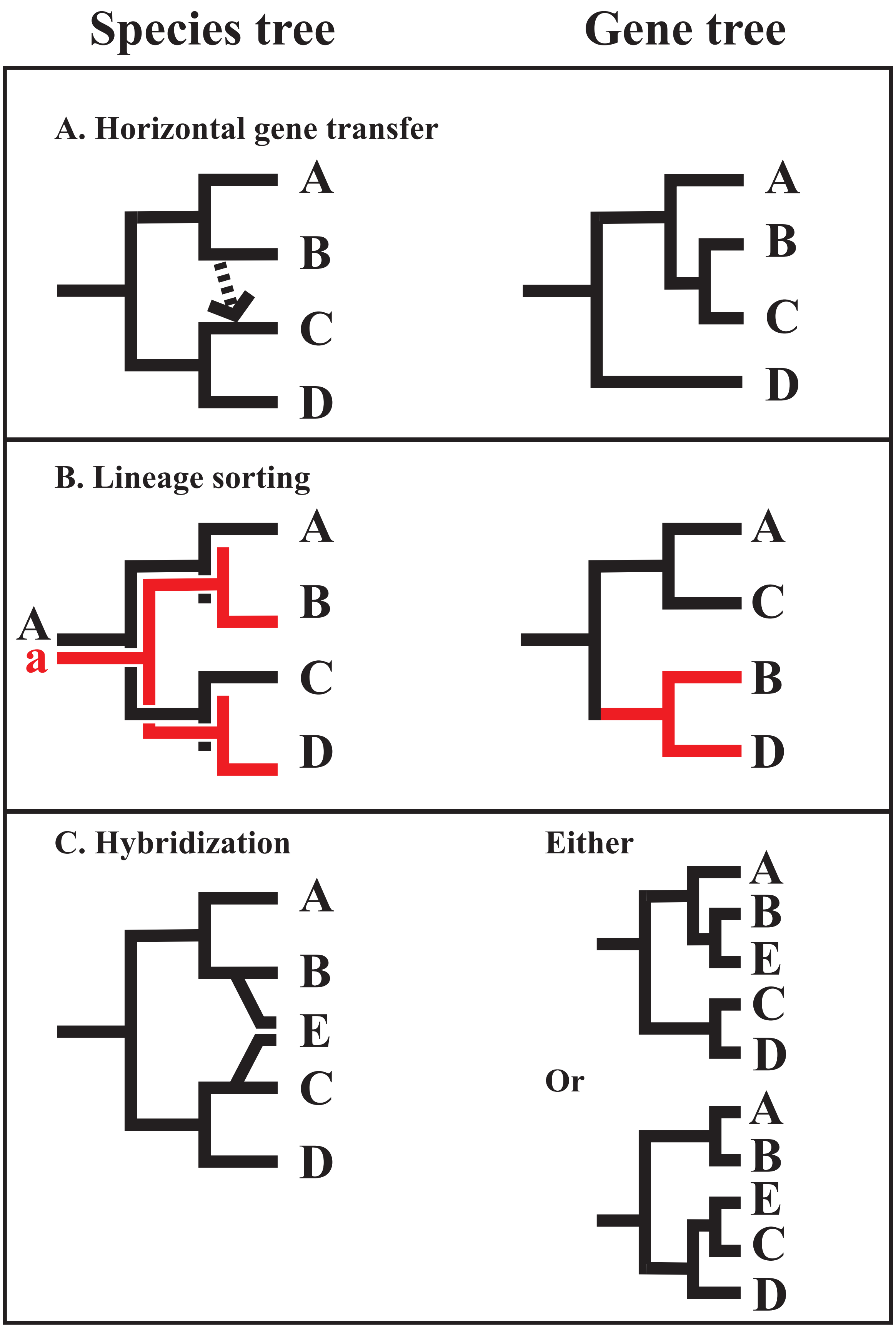

A
Cosmarium verrucosum JH0068
Euastrum crassa var. michiganense JH0018
Xanthidium hastiferum JH0054
Pleurotaenium contrictum JH0135
79
-
-
Micrasterias foliaceae NIES 297
58
-
Triploceras gracile JH0215
100
Micrasterias truncata JH0106
Micrasterias radiata 226
Micrasterias muricata JH0179
10 changes
B
Euastrum crassa var. michiganense JH0018
Pleurotaenium contrictum JH0135
Cosmarium verrucosum JH0068
Xanthidium hastiferum JH0054
Triploceras gracile JH0215
Micrasterias muricata JH0179
Micrasterias foliaceae NIES 297
Micrasterias radiata 226
Micrasterias truncata JH0106
C
Micrasterias radiata 226
Micrasterias truncata JH0106
Cosmarium verrucosum JH0068
Micrasterias foliaceae NIES 297
100
Triploceras gracile JH0215
-
-
-
-
Xanthidium hastiferum JH0054
74
Micrasterias muricata JH0179
Euastrum crassa var. michiganense JH0018
Pleurotaenium contrictum JH0135
0.01 substitutions per site
D
Pleurotaenium contrictum JH0135
Euastrum crassa var. michiganense JH0018
Cosmarium verrucosum JH0068
Micrasterias muricata JH0179
1.0
0.83
0.55
1.0
Micrasterias truncata JH0106
0.89
Xanthidium hastiferum JH0054
Micrasterias radiata 226
Triploceras gracile JH0215
Micrasterias foliaceae NIES 297
0.05 substitutions per site

Figure 7.6. Model genealogies showing potentially confounding evolutionary events.


Species tree
Gene tree
A. Horizontal gene transfer
A
B
C
D
A
B
C
D
B. Lineage sorting
A
a
A
B
C
D
A
C
B
D
C. Hybridization
A
B
E
C
D
Either
A
B
E
C
D
Or
A
B
E
C
D

Chapter 8. Conclusions

Experimental conclusions

In the completion of this work, two molecular markers for estimation of phylogenetic relationships among the conjugating green algae were developed. Hundreds of new strains were isolated that will be available for future investigations. This study of the phylogenetic relationships among conjugating green algae revealed that our concepts of the families and genera in the Zygnematales requires substantial revision. Besides that the Zygnematales appears to be paraphyletic (Chapter 3), it is clear that the families and most of the unicellular genera of the Zygnematales are not monophyletic. These certainly merit more careful investigation using both cytological and molecular phylogenetic methods. Although most families in the Desmidiales are monophyletic, the family Peniaceae (and the genus *Penium*) is not supported as monophyletic based on the collected data (Chapter 3) and the desmid lineage almost certainly includes or is closely related to the zygnematalean genus *Roya* (Chapter 3, 5).

These data support the use of cytological features as systematic characters in some cases. The separation of the genus *Heimansia* from *Cosmocladium* and *Haplotaenium* from *Pleurotaenium*, decisions made based on structural characteristics, was supported in these analyses (Chapter 3). These data also suggest that chloroplast position may be a useful systematic character as this characteristic sets apart major lineages within the Zygnematales and separates these organisms from basal desmids (Chapter 3). These data also indicate that the genera *Mesotaenium*, *Cylindrocystis*, *Cosmarium*, *Staurodesmus*, *Penium*, *Hyalotheca*, *Desmidium* and *Spondylosium*, are not monophyletic.

In some cases, these phylogenetic relationships can be correlated to differences in ontogeny or cellular structure. In the case of the filamentous Desmidiaceae, a suite of cell division characteristics as well as chloroplast shape characterized the clades of the polyphyletic *Desmidium* and *Spondylosium* (Chapter 4, 5). Cell division in *Desmidium pulchrum*, *Isthmocatena pulchella*, *Spondyosium tetragonum* and *Teilingia granulata* was described for the first time. This led to the systematic reassignment of several taxa and the creation of a new generic name, *Isthmocatena* (Chapter 5). This implies that critical cellular processes such as cell division can vary and the differences discovered were apparent changes in the timing and order of cellular events as well as the evolution of a novel structure.

Sometimes the reason for phylogenetic ambiguity is less obvious. In the case of the Gonatozygaceae, organisms that were assigned to the same species based on their gross structural characteristics were phylogenetically distinct (Chapter 6). Careful attention to the structural characteristics was sufficient to distinguish most clades, but in the case of *Gonatozygon brebissonii*, it was not possible to distinguish among the different lineages even using EM techniques (Chapter 6). In this case the structural simplicity of organisms can mask their phylogenetic diversity. This poses a difficult challenge when interpreting any study based on the morphological identity of structurally simple microorganisms. The results of this study suggest that we may be underestimating phylogenetic and possibly species diversity in groups of structurally simple organisms.

However, one must be careful in the interpretation of certain characteristics. Investigaton of *Triploceras* suggests that even fundamental characteristics such as apparent symmetry may be variable among closely related organisms (Chapter 7). Never before had a relationship between *Micrasterias* and *Triploceras* been proposed. Knowledge of this relationship forces us to think about the utility of symmetry as a taxonomic character, and encourages us to think more critically about the anatomy of these organisms. In this case, the relationship opens up a number of possible research avenues into the basic biology of these organisms, their morphogenesis and their evolution.

Towards an understanding of diversity in the conjugating green algae and its application to the origin of land plants

In general, caution when interpreting the structure of microorganisms must be observed. Structural characteristics must be investigated in a systematic context if we are to understand their origin, evolution and systematic utility. While molecular phylogenetic investigations should continue, data collected in this work suggest that there is a wealth of systematic information hidden in the structure and ontogeny of microorganisms. Future investigations can use careful observation in a phylogenetic context to begin to understand the evolution of microorganisms.

These data also suggest that both the developmental and phyletic diversity of conjugating green algae are probably underestimated. While this poses some systematic and taxonomic challenges, it also suggests that the conjugating green algae may be a richer source of evolutionary information than was previously suspected. This work suggests that the conjugating green algae would be excellent models for the study of cellular morphogenesis, cell division, and cell wall synthesis. It is possible that there are also genetic and genomic characteristics shared between the conjugating green algae and land plants that have contributed to their success.

It is not clear which lineage of charophyte green algae is most closely related to land plants. However, the Characeae, Coleochaetales and the conjugating green algae are all possible candidates. Among these, the conjugating green algae are the most structurally and developmentally diverse and can provide insight into the evolution of these characteristics that other lineages cannot.

Shifts in body plan are considered important evolutionary changes in the history of complex forms such as land plants. The conjugating green algae are all strongly axile, having a single main axis and cell division occurs in only one plane. However, all conjugating green algae are capable of site-specific modification of the cell wall. Formation of conjugation tubes, gamete pores, and rhizoids as well as secretion of extracellular polysaccharides are carefully controlled. Most conjugating green algae seem to have taken a different evolutionary path than *Spirogyra*, the earliest branching lineage of the conjugating green algae (Chapter 2). In most Zygnematophytes, the cytoplasmic contents are based in the center of the cell, however, in *Spirogyra* (including *Sirogonium*) only the nucleus is central and the other cytoplasmic contents are appressed to the outer membrane by a large central vacuole. In this respect, *Spirogyra* is organized more like *Coleochaete* and *Klebsormidium* than most desmids, with the exception of the placement of the nucleus. In these latter taxa, the nucleus is also peripheral. It is noteworthy that the earliest diverging lineages of zygnematophytes contain filamentous organisms. Because the most closely related charophyte algae are also filamentous, this is likely to be a plesiomorphic trait and the unicellular condition of some zygnematophytes may, therefore, be derived.

Early diverging lineages of zygnematophytes contain organisms that are structurally diverse and developmentally complex. All conjugating green algae have zygotic meiosis. However, many desmids are thought to be asexual, some are parthenogenic, and zygotes may result from various sexual strategies: anisogamy (oogamy?), isogamy, homothallism, heterothallism, etc. (Brook, 1981). We do not fully understand the processes involved in sexual reproduction. It is known that at least one pheromone is involved in the induction of sexual cell division in *Closterium* (Fukumoto et al., 2003b). It is now possible to begin to understand the phylogenetic relationships among these organisms and speculate on their evolutionary significance.

Even within the critical process of cell division, this research has shown that many variations are possible. The discovered differences were not only modifications of existing processes, but involved the evolution of novel structures. Additionally, these variations were found among some of the structurally least complex members of the Desmidiaceae, suggesting that apparent structural diversity in interphase cells does not necessarily correlate to overall developmental complexity.

Estimation of ancestral characteristics is never a trivial undertaking and diversity in the conjugating green algae makes such assignments particularly difficult. What is clear is that the conjugating green algae and probably their last common ancestor are structurally, developmentally, and reproductively complex. This implies that the ancestor of the zygnematophytes and the lineage that gave rise to land plants were probably also complex organisms. This innate complexity and the possible structural, developmental, reproductive and presumably genetic diversity may have allowed one lineage to successfully colonize the land and the other to become dominant members of many freshwater and subaerial habitats.

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