ABSTRACT

Title of Thesis: EVOLUTIONARY TRANSITIONS BETWEEN
                          STATES OF STRUCTURAL AND
                          DEVELOPMENTAL CHARACTERS AMONG
                          THE ALGAL CHAROPHYTA
                          (VIRIDIPLANTAE).

                          Jeffrey David Lewandowski, Master of Science,
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The Charophyta comprise green plant representatives ranging from familiar

complex-bodied land plants to subtle and simple forms of green algae, presumably

the closest phylogenetic relatives of land plants. This biological lineage provides a

unique opportunity to investigate evolutionary transition series that likely facilitated

once-aquatic green plants to colonize and diversify in terrestrial environments. A

literature review summarizes fundamental structural and developmental transitions

observed among the major lineages of algal Charophyta. A phylogenetic framework

independent of morphological and ontological data is necessary for testing hypotheses

about the evolution of structure and development. Thus, to further elucidate the

branching order of the algal Charophyta, new DNA sequence data are used to test

conflicting hypotheses regarding the phylogenetic placement of several enigmatic

taxa, including the algal charophyte genera *Mesostigma*, *Chlorokybus*, *Coleochaete*,

and *Chaetosphaeridium*. Additionally, technical notes on developing RNA methods

for use in studying algal Charophyta are included.

EVOLUTIONARY TRANSITIONS BETWEEN STATES OF STRUCTURAL AND
DEVELOPMENTAL CHARACTERS AMONG THE ALGAL CHAROPHYTA
(VIRIDIPLANTAE).

By

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Preface

The following are examples from several of the works that have provided philosophical inspiration for this document.

"Nothing in biology makes sense except in the light of evolution."
Theodosius Dobzhansky
American Biology Teacher, volume 35: 125-129, 1973

"Body form evolves through geologic time, but it also arises in each generation through development. The time frames and apparent processes couldn't be more different, but explanations of the evolution of form have to consider how form is generated, and they must account for both underlying stability and immense change in design."
Rudolf A. Raff
The Shape of Life: Genes, Development, and the Evolution of Animal Form, 1996

"There is a parallel, long appreciated, between the developmental changes that convert an egg into an adult, and the evolutionary changes that, on an enormously longer time scale, have converted simple single-celled ancestors into the existing array of multicellular animals and plants."
John Maynard Smith
Shaping Life: Genes, Embryos and Evolution, 1999

"Developmental biologists must come to appreciate diversity, the comparative method, and the importance of phylogeny. Evolutionary biologists need to understand and incorporate the underlying rules of developing systems into a more comprehensive theory of evolution."
Rudolf A. Raff
The Shape of Life: Genes, Development, and the Evolution of Animal Form, 1996

"The body of a plant can best be understood in terms of its long history and, in particular, in terms of the evolutionary pressures involved in the transition to land."
Peter H. Raven, Ray F. Evert, and Susan E. Eichhorn
Biology of Plants, 1999

Dedication

This work is dedicated to my mother, Nancy, and my father, Jerome, who have supported my education and always encouraged me to pursue my love of science. It is also dedicated in loving memory to Jane Kulas, my grandmother, and to the memory of Dr. Tzong-Yow "T.Y." Lee, one of the finest and most dedicated teachers I have encountered.

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throughout my life.

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Chapter 1: Introduction

Consider the 4.5 billion-year history of planet Earth. Each extant species on our planet is the product of more than 3,500 million years of evolutionary change acting upon generations of life forms, all presumed to share a common ancestor. Thus, for most of the course of Earth's history, the dynamical processes of physical change and organismal evolution have shared an intimate linkage; geologic transformation has played a part in shaping biological forms and, likewise, biological evolutionary change has had a significant impact on physical geography. Some evolutionary events of inherently botanical nature—the origin of photosynthesis, the acquisition of primary plastids through endosymbiosis, and the colonization of terrestrial environments by plants—have had especially profound effects on the biosphere of our planet. A thorough understanding of the evolutionary origin of land plants, chronologically the most recent of these historical examples, requires an examination of the morphologically simpler green algal forms most closely related to the more familiar land plant lineage.

Due to their awesome environmental, ecological, and economic importance, arguably no group of multicellular organisms is more vital for humans to understand than the land plants, also called embryophytes. Both labels—'embryophytes' and 'land plants'—possess the potential to confound the biology of the group they identify (Graham, 1993). For the sake of this document, both terms, land plants and embryophytes, will be used interchangeably as synonyms, defined as the group including the bryophytes plus the tracheophytes. A number of studies utilizing

different lines of evidence support the monophyly of the land plant clade (Mishler et al., 1994; Waters and Chapman, 1996; Lewis et al., 1997).

To even the casual observer or amateur naturalist, a close relationship between land plants and green algae lies well within the realm of imagination. Predictions about the exact nature of such a relationship have existed for over a century (Bower, 1908). Fossils representing the oldest known embryophyte remains indicate the colonization of land by plants occurred more than 470 million years ago (Gray et al., 1982). To date, no discovery of older embryophyte fossils has been made, so there exists no direct physical evidence revealing the relationship between green algae and land plants (Graham, 1993; Graham and Wilcox, 2000). Thus, hypotheses about evolutionary relationships, both between algae and land plants and among the aquatic and terrestrial groups comprising this monophyletic clade, must be drawn by application of the comparative method to data collected from extant green algal and embryophyte entities.

Comparative studies of morphology have been used to infer phylogeny in various biological groups. Homoplasy, such as convergence of forms through parallel evolution, is quite prevalent in the green algae, and in the algae in general (Graham and Wilcox, 2000). This phenomenon confounds attempts to infer phylogeny based on external morphology alone (Pickett-Heaps and Marchant, 1972; Pickett-Heaps, 1975; Graham and Wilcox, 2000). Numerous biochemical (Frederick et al., 1973; Syrett and al-Houty, 1984; DeJesus et al., 1989; Jacobshagen and Schnarrenberger, 1990; Gross, 1993) and ultrastructural (Pickett-Heaps and Marchant, 1972; Pickett-Heaps, 1975; Cook et al., 1998) investigations have

contributed to the resolution of evolutionary relationships between green algae and land plants. Based on consideration of ultrastructural, biochemical, and morphological characters, Mattox and Stewart (1984) named a new class of green algae, the Charophyceae, grouping the lineages hypothesized to be most closely related to embryophytes.

The Charophyceae *sensu* Mattox and Stewart (1984) include five orders; they are, in likely order of evolutionary divergence, the Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales, and Charales. A problem with this taxonomic group, however, is that it does not include the land plants. Thus, if the embryophyte lineage is assumed to have diverged from within the charophycean green algae, the Charophyceae *sensu* Mattox and Stewart (1984) is paraphyletic, an unnatural group that does not reflect evolutionary relationships. Bremer (1985) recognized this dilemma and advised recognition of the charophycean algae plus embryophytes as a novel division, the Streptophyta, distinct from all other green algae. A number of phylogenetic analyses of molecular sequence data (Chapman and Buchheim, 1991; McCourt et al., 1996; Karol et al., 2001; Turmel et al., 2002b) and several genome architectural characters (Baldauf et al., 1990; Manhart and Palmer, 1990; Starke and Gogarten, 1993) support Bremer's (1985) proposed classification. Additionally, actin gene sequence data (Bhattacharya et al., 1998) support the inclusion of a scaly, biflagellate, unicellular alga, *Mesostigma viride*, at the base of the Streptophyta lineage. Utilizing a multi-gene dataset representing nuclear, chloroplast, and mitochondrial DNA characters, molecular sequence analyses by Karol et al. (2001) corroborate this finding; authors of the study proposed the name Charophyta for the

phylogenetic lineage comprising land plants, charophycean green algae (the Charophyceae *sensu* Mattox and Stewart, 1984), and *Mesostigma*.

Based on all evidence to date, the "green plant" lineage, Viridiplantae *sensu* Cavalier-Smith, (1981), splits into two major branches; the Charophyta *sensu* Karol et al. (2001) include land plants and their green algal relatives, and the Chlorophyta comprise the ulvophycean, trebouxiophycean, chlorophycean, and most of the yet-to-be classified flagellate (prasinophyte) green algae. This taxonomic scheme, with one exception, will be exercised for the remainder of this treatment. Due to conflicting data on the phylogenetic placement of the enigmatic genus *Mesostigma*, and the importance of considering all possible evolutionary hypotheses, the author will refrain from assuming inclusion of this genus in the Charophyta. Here, the genus *Mesostigma* will be treated as a currently unallied entity. Forthcoming discussion will use the term Charophyta, or the charophytes, to refer to land plants plus the closely related green algae of the Charophyceae *sensu* Mattox and Stewart (1984). Furthermore, to avoid confusion associated with naming paraphyletic groups, the lineages of green algae traditionally termed charophyceans (Graham, 1993; Graham and Wilcox, 2000; Graham et al., 2000), or the Charophyceae (*sensu* Mattox and Stewart, 1984) will be referred to here as the algal Charophyta, algal charophytes, algal members of Charophyta, and so forth.

According to Raven et al. (1999) "the body of a plant can best be understood in terms of its long history and, in particular, in terms of the evolutionary pressures involved in the transition to land." These conditions for comprehension of the plant body require consideration of both algal members and land plant representatives of

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the Charophyta. Regarding evolutionary history, molecular systematic analyses of a four-gene DNA sequence dataset have revealed phylogenetic relationships among algal groups within Charophyta at an unprecedented level of resolution (Karol et al., 2001). Utilizing data from each of the three genomes of the green plant cell, Karol et al. (2001) found the Charales to be the extant algal group most closely related to embryophytes. Additionally, the phylogeny presented bolsters support for many of the other relationships among the algal Charophyta. Some of these relationships are congruent with findings based on analysis of chloroplast small- and large-subunit ribosomal DNA (Turmel et al., 2002b). These results are exciting and contribute much to our understanding of evolution in the Charophyta. However, because phylogenetic problems can never be deterministically solved, trees generated by such analyses should be treated as hypotheses that are to be tested and retested with new data and ever-improving probabilistic methods. Two analyses included in this report utilize previously unavailable data to test phylogenetic hypotheses, specifically those involving the enigmatic genera *Mesostigma* and *Chaetosphaeridium*.

In examining adaptations necessary for the transition of green plants to terrestrial environments, consideration of the algal Charophyta is inherently vital. At all levels of land plant body organization—macroscopic to microscopic, physiological, cellular, and molecular—adaptations to terrestrial life have been investigated extensively (Kenrick and Crane, 1997). Some of the most fundamental innovations associated with colonization of the land originated in, and thus were likely inherited from, the algal ancestors of modern land plants (Graham, 1993). Yet, relatively few investigators have studied the evolution of specialized body features

and cellular mechanisms in the algal Charophyta, the lineage that would include algal ancestors of embryophytes (if such ancestors were present today) and does comprise the extant taxa most likely to resemble those ancestors. Due to the generally microscopic nature of green algal features, such characters are sublime relative to many of the traditionally studied land plant body parts. Because characters relevant to the invasion of land are plesiomorphic with respect to the embryophyte clade, describing the evolution of many character states is not possible without consideration of the algal Charophyta. Admittedly, botanists have utilized green algal experimental systems in pursuit of a better understanding of land plant form and function. For many decades *Acetabularia*, *Chlamydomonas*, and *Chlorella* have been used in investigations of plant cytology, morphology, and development. Because these and other commonly studied genera of green algae are now known to have diverged from the Chlorophyta lineage of the Viridiplantae, structures that have evolved in these algae are not homologous to similar features found in land plants. While these studies have contributed to the advancement of knowledge about evolution among chlorophyte green algae, it is illogical to extend findings across branches of the tree of life. Thus, such results are not informative to those inquiring about the evolution of morphology and development in the embryophyte and algal representatives of the Charophyta. Understanding of morphology and development in land plants is therefore incomplete without consideration of the green algal members of the Charophyta. Future investigators should seriously consider more extensive utilization of algal charophyte experimental systems in studies of the evolution and development of land plants and related organisms.

Incorporation of information about the algal Charophyta is clearly a key to understanding the plant body. The evolution of morphology and development across the algal Charophyta is also intrinsically fascinating. Although the green algae lack the extraordinary morphological complexity and diversity of land plants, the algal Charophyta exhibit an impressive array of structural and mechanical characteristics with an exceptionally interesting pattern of phylogenetic distribution (Graham, 1993; Graham and Wilcox, 2000). *Mesostigma viride* is a scaly, unicellular biflagellate that likely diverged from the base of the Charophyta (Melkonian and Surek, 1995; Bhattacharya et al., 1998; Karol et al., 2001) or at the base of the Viridiplantae, prior to the split distinguishing the Charophyta and Chlorophyta lineages (Lemieux et al., 2000; Turmel et al., 2002a; Turmel et al., 2002b). Members of the Charales and Coleochaetales exhibit multicellular parenchyma, phragmoplastic cytokinesis, plasmodesmata, oogamous sexual reproduction, differentiated reproductive structures, apical growth, and a number of other highly derived characteristics reminiscent of fundamental land plant features. Other groups of algal Charophyta, diverging between the Mesostigmatales and Charales branches, exhibit a variety of forms—colonial or sarcinoid, simple filamentous, branched filamentous and parenchymatous—and numerous types of specially differentiated cells with distinct functions. In contemplating the evolution of morphological transitions between lineages of the algal Charophyta, from unicellular to more and more complex multicellular forms, it becomes difficult to ignore a striking similarity between those evolutionary changes and the ontogenetic stages observed in more derived examples of algal charophyte thallus development. Unicellular, often flagellate stages develop

into simple filaments, and then into branched filaments or parenchyma before some cells differentiate for specialized functions. The Charophyta thereby represent a valuable "model lineage" within which the intimately related phenomena of evolution and development may be explored.

This concept for approaching investigation of the Charophyta is actually a fresh reinvention of an old idea, a synthesis of evolutionary and developmental biology. Evolutionary developmental biology, or evo-devo, refers to the combination of two historically distinct disciplines in biology in order to pursue a grander understanding of evolution. Morphological and mechanical features of organisms originate and then change over long periods of geologic time through the process of biological evolution. Those features, including every existing variation, also arise via developmental mechanisms in each and every life-history generation of the organisms in which they evolved. Raff (1996) describes this close relationship between evolutionary and developmental processes, and recommends several directions for intertwining the two long-distinct biological disciplines. According to Raff (1996), developmental biologists need to value and understand biodiversity, the comparative method, and the importance of testable phylogenies; likewise, evolutionary biologists, in order to generate more complete evolutionary hypotheses, must come to understand how developing systems function. The literature review, experimental results, and technical methodology to follow strive to incorporate the spirit of this endeavor, the synthesis of evolutionary and developmental thought, into an investigation of the evolution of form and function in the Charophyta, one of the most important and interesting branches on the tree of life.

This thesis has three main objectives: a review of cytology, morphology, and development in representatives of the algal Charophyta; the reporting of results that contribute new data to the task of resolving some of the more controversial phylogenetic relationships among the green algae of the Charophyta lineage; and description of technical progress in developing RNA methods for eventual examination of molecular mechanisms that likely underlie the morphological and developmental transitions observed in the Charophyta.

Chapter 2: The Algal Charophyta

In order to elucidate the evolutionary transitions between lineages of the algal Charophyta, the following literature review aims to describe the cytological, morphological, and developmental features that characterize each of the five recognized orders of algal charophytes, as well as the related taxon, *Mesostigma*. Complexity at all levels generally increases from earlier to later diverging lineages in the Charophyta. This justifies the following approach, in which discussion of taxa with simpler morphology will include more details about cytological features, with emphasis on characters that define the Charophyta in general. Likewise, the more derived thalli of later-diverging taxa will provide ample opportunity for description of complex morphology and the development of specialized cells and multicellular structures. To best demonstrate evolutionary and developmental trends, organisms will be treated in the order of phylogenetic divergence, as it is currently best understood (*sensu* Karol et al., 2001).

2.1   Mesostigmatales

Mesostigma viride Lauterborn is the only representative of the monogeneric order Mesostigmatales that has been studied with modern methods. *Mesostigma* had traditionally been classified among the morphologically simple prasinophycean green algae, unicellular flagellates classified into a group that is now recognized as unnatural. Based on ultrastructural data, Rogers et al. (1981) first predicted a close phylogenetic relationship between *Mesostigma* and the charophyte lineage. Subsequent ultrastructural analyses (Melkonian, 1982; 1984; 1989) supported the

hypothesis including *Mesostigma* among the algal charophytes. With advances in

molecular methods for collecting nucleic acid sequence data and systematic methods

for conducting phylogenetic analyses of such datasets, a way to test this evolutionary

hypothesis using characters independent of the morphology and ultrastructure in

question became possible. Yet, the exact phylogenetic position of the

Mesostigmatales is still controversial. Inclusion of *Mesostigma* within the

Charophyta clade has been supported by analyses of sequence data from nuclear

small subunit ribosomal RNA (SSU rRNA) genes (Melkonian and Surek, 1995;

Marin and Melkonian, 1999), nuclear actin genes (Bhattacharya et al., 1998), and a

combined analysis of nuclear SSU rRNA, chloroplast, and mitochondrial genes

(Karol et al., 2001). Contrary to these results, analyses of sequence data from

chloroplast (Lemieux et al., 2000; Turmel et al., 2002b) and mitochondrial (Turmel et

al., 2002a) genomes support placement of the Mesostigmatales at the base of the

Viridiplantae, prior to the divergence of the two major lineages, Chlorophyta and

Charophyta, *sensu* Karol et al. (2001). Incongruence between various molecular

phylogenetic studies may be due to inadequate taxon sampling (Hillis, 1998; Qiu and

Lee, 2000; Karol et al., 2001; Pollock et al., 2002). Despite the current debate over

its phylogenetic position, there is little doubt that *Mesostigma* likely diverged early in

the Charophyta lineage or at the base of the Viridiplantae, before the splitting of the

two major groups of green plants. In either case, *Mesostigma* is likely to share some

ancestral character states with members of the charophyte lineage. Likewise, many of

the putative characteristics of a green flagellate ancestor of the charophytes proposed

by Graham (1993) are present in *Mesostigma*. For these reasons, any treatment of

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morphology and ontogeny in the algal Charophyta is well served by an early discussion of *Mesostigma*.

Mesostigma is presumed to be a motile unicell primarily encountered in freshwater habitats (Graham and Wilcox, 2000). Cells of *Mesostigma* take the form of warped disks with two flagella emerging laterally from a deep flagellar pit opening (Sym and Pienaar, 1993). Lacking a cell wall, *Mesostigma* cells are covered by at least three distinct scale layers. The outermost layer consists of large, ornate, basket-like scales; middle layer scales are smaller, flat, oval in shape, and decorated with pits (Manton and Ettl, 1965). Most similar to the scales observed in flagellate cells of the reproductive stages of many charophytes (*Chlorokybus*, *Coleochaete*, *Chaetosphaeridium*) are innermost scales covering *Mesostigma*—small, flat, and ranging from square- to maple leaf-shaped (Marin and Melkonian, 1999; Graham and Wilcox, 2000). Between the innermost scale layer and the cell is a substance associated with both the scales and the cell membrane. This glue-like material has been hypothesized to represent an evolutionary precursor to charophyte cell walls (Rogers et al., 1980).

Mesostigma cells contain a single chloroplast, shaped like a shallow bowl with regions at the margin thicker than in the center (Sym and Pienaar, 1993). Plastids contain several pyrenoids and, unlike typical charophyte chloroplasts, an eyespot composed of several globular pigment layers, usually located near the flagellar basal bodies. Associated with the basal bodies and situated between the plastid and flagellar apparatus is a lobed microbody, a structure also referred to as a peroxisome (Rogers et al., 1981). Each basal body is associated with two microtubular roots.

The "s" root consists only of microtubules, but the "d" root is composed of several layers—an array of microtubules, a thin series of parallel plates, several amorphous regions, and an arrangement of smaller tubules. Such multilayered structures (MLSs) are typical of roots in the flagellate cells of both algal and land plant representatives of the charophytes, although each *Mesostigma* cell contains two MLSs and flagellate cells of charophytes possess only one. Basal bodies in *Mesostigma* are linked by connecting fiber, material that appears densely stained in transmission electron microscopy (TEM). Flagella are approximately equal in length to the cell and are covered by small polygonal scales similar to those that make up the innermost layer of cell body scales.

At present, *Mesostigma* appears to be the most sensible experimental system for studying features of the presumed green flagellate ancestor of the charophyte lineage. In addition to the ultrastructural, biochemical, and phylogenetic studies that have been conducted, complete DNA sequences of the *Mesostigma* chloroplast and mitochondrial genomes have recently been published (Lemieux et al., 2000; Turmel et al., 2002a). However, a number questions about this mysterious organism remain unanswered.

Although most reported collections of *Mesostigma* specify a freshwater habitat, a recent environmental sampling study of coral reefs in the southern Caribbean reported a puzzling result. Utilizing 16S chloroplast SSU rDNA markers, two clones from the marine samples resulted in sequences that when BLAST-searched return 89% and 92% identities to the *Mesostigma viride* chloroplast DNA sequence (Frias-Lopez et al., 2002). With members of the Charophyta currently

known only to inhabit freshwater and terrestrial environments, an obvious question arises: Are marine examples of the Mesostigmatales or closely related taxa awaiting discovery and description?

Description of sexual reproduction in *Mesostigma* has not been published, yet distinct mating types are listed in one of the mainstream culture collections (NIES). Non-motile *Mesostigma* cells are common in culture (Lewandowski, unpublished results), but whether these represent yet-to-be described life history stages or responses to environmental pressures is currently unknown. Published accounts of *Mesostigma* life history are generally incomplete and inconsistent. Much work remains to be done in this area.

Furthermore, the phylogenetic relationships between *Mesostigma* and other flagellate green algae and protists are not well understood, as relatively few unicellular eukaryotes have been included with *Mesostigma* in molecular phylogenetic studies.

2.2   Chlorokybales

The order Chlorokybales (Mattox and Steward, 1984) is composed of a single described species, *Chlorokybus atmophyticus*. *Chlorokybus* is currently thought to represent the earliest diverging lineage of algal charophytes exhibiting a non-motile, multicellular vegetative thallus life history stage (Graham and Wilcox, 2000). This conjecture is supported by recent analyses of combined DNA sequences from the chloroplast, mitochondrial, and nuclear genomes of algae representing most of the major charophyte lineages (Karol et al. 2001). While little is known about

Chlorokybus ecology, its habitat is presumed to be terrestrial or freshwater and it can be grown in culture using either solid agar or liquid medium (Lewandowski, unpublished results). Despite reports that *Chlorokybus* has only rarely been collected and isolated (Rogers et al., 1980), strains that have not yet been studied with modern methods seem to be available from reputable culture collections.

Sexual reproduction has not been observed in *Chlorokybus*, but asexual propagation occurs via zoosporogenesis. Each cell of the vegetative thallus may produce a single biflagellate zoospore. Interestingly, some characteristics of *Chlorokybus* zoospores, as revealed by an ultrastructural study conducted by Rogers et al. (1980), are similar to those of *Mesostigma* cells and the motile cells of other charophytes. The ovoid zoospores of *Chlorokybus*, like cells of *Mesostigma*, feature two laterally inserted flagella and a flagellar groove. Flagella are scaly, like those of *Mesostigma*, and also possess hairs similar to those of other algal charophyte flagella. Such flagellar hairs are not found in *Mesostigma* (Marin and Melkonian, 1999; Graham and Wilcox, 2000). Like the flagellate cells of other charophytes, the *Chlorokybus* zoospore is wall-less, but instead is covered by small flat scales, reminiscent of those found in the innermost layer of the *Mesostigma* cell covering.

Zoospores contain a single cup-shaped chloroplast. Within the chloroplast are two distinct types of pyrenoid. Embedded within the plastid is a very large pyrenoid characterized by traversing thylakoids. Found at the periphery of the chloroplast are one or more smaller pyrenoids that lack thylakoids. These enigmatic structures are sometimes termed "pseudopyrenoids." Distinct from *Mesostigma*, yet similar to motile cells of other algal charophytes, the *Chlorokybus* zoospore possesses a plastid

lacking an eyespot. Zoospores also contain a single cup-shaped microbody (peroxisome), attached at its narrow base to the flagellar apparatus and surrounding a portion of the nucleus with the broader, hollowed end. The two mitochondria observed in *Chlorokybus* zoospores are also attached to the flagellar apparatus. The flagellar apparatus includes two basal bodies, linked by connecting fiber, and a microtubular root associated with an MLS. The MLS of *Chlorokybus* zoospores is similar to those found in *Mesostigma* and the motile cells of some charophyte genera, yet is distinct in several ways (Rogers et al., 1980). Unlike other MLS roots found in this lineage, the segmented layer extends beyond the rest of the root. The entire root generally extends a relatively short distance into the cell, rarely reaching the depth of the nucleus. Additionally, the *Chlorokybus* MLS has been described as narrow, composed of only 10-11 microtubules, although there is some evidence that the number of root microtubules may be variable.

After swimming for approximately an hour, zoospores cease motion. Germination initiates as flagella are retracted at the point of insertion and the cell becomes more spherical. Completing the solitary vegetative cell, a cell wall is secreted between the cell membrane and layer of scales. The scaly layer is eventually shed. Internal cytology of vegetative *Chlorokybus* cells is quite similar to that of the zoospores (Lockhorst et al., 1988). Interphase cells feature a nucleus, with central nucleolus, lying between a single large plastid and a lone microbody. The parietal chloroplast lines nearly half the inner surface of the cell and contains two distinct pyrenoids—one large central pyrenoid, coated with starch granules and traversed by single thylakoids, and a smaller peripheral "pseudopyrenoid," lacking thylakoids and

starch coating (Lockhorst et al., 1988). Mitochondria and dictyosomes are sparsely

distributed throughout the cytoplasm; ribosomes and endoplasmic reticula are quite

abundant. Closely associated with the nucleus, the microbody is also attached to a

pair of centrioles. Developmental precursors of flagellar basal bodies, the two

centrioles are linked by a structure resembling the connecting fiber that link basal

bodies in the zoospore flagellar apparatus.

To form the sarcinoid thallus characteristic of *Chlorokybus*, solitary vegetative

cells undergo a series of cell divisions. In cell division of true unicellular algae, new

cell wall material is deposited completely surrounding the entire surface of each

daughter cell. Contrary to this, *Chlorokybus* is considered to incorporate true

vegetative cell division (Bold and Wynne, 1978; Rogers, 1980) because cytokinesis

involves the deposition of a septum-like cross wall that divides the parental cell into

two compartments. Early events of cell division include cleavage of the pyrenoids

and division of the plastid at a plane that is marked by cell membrane invagination,

indicative of the future site of cytokinesis. At prophase the centrioles migrate along

arrays of microtubules from their early-mitotic position, associated with the nucleus

and microbody at the plane of division, to the poles of the forthcoming spindle.

Mitosis is open, with disruption of the nuclear membrane, from prometaphase

through telophase. Chromosome separation is seemingly facilitated by drastic

elongation of the spindle during anaphase, and spindle microtubules are persistent

until completion of telophase, presumably aiding in the separation of the two

telophase daughter nuclei (Lockhorst et al., 1988). Early mitotic furrowing of the cell

membrane, open mitosis, and spindle elongation and persistence are all characters of

mitosis observed in other algal charophytes, but not found in members of the Chlorophyta lineage (Graham and Wilcox, 2000). Completion of the membrane partitioning the two daughter cells occurs with fusion of vesicles and continued furrowing along a transverse array of microtubules. A septum-like cell wall common to the two daughter cells is secreted, completing cytokinesis. Plasmodesmata are not present in the *Chlorokybus* cross-wall.

Division of a solitary *Chlorokybus* vegetative cell forms a 2-celled arrangement, shaped like a short cylinder with rounded ends. The next division creates a four-celled array like a square (2 x 2) with rounded corners. Most *Chlorokybus* thalli are composed of 2-4 cells, yet older, larger arrays of cells are possible through additional divisions. For example, a third serial division results in a blunt-cornered, cube-shaped (2 x 2 x 2) packet of eight cells. Clearly, the term "sarcinoid," loosely defining thalli with vegetative growth not resulting in a filament or parenchyma (Bold and Wynn, 1978; Rogers et al., 1980), can refer to thalli of various shapes and sizes. All vegetative configurations are encased in a thick sheath of extracellular matrix material, or mucilage.

Each cell of the *Chlorokybus* thallus is capable of producing a single zoospore. After the protoplast is transformed into a scaly zoospore, flagella commence beating while still within the confines of the parental cell wall. Crude dissolution of the cell wall allows for release of the zoospore, thus renewing the cycle of asexual propagation and dispersal.

2.3   Klebsormidiales

Following *Chlorokybus* on the phylogenetic lineage of algal Charophyta, the Klebsormidiales are the next diverging order of morphologically simple green algae. *Klebsormidium* (formerly *Hormidium*) was once allied with members of the Ulotrichales (Chlorophyta) based on superficial similarities observed at the light microscope level of resolution. This hypothesized relationship was challenged due to striking differences between patterns of mitosis and cytokinesis in the two genera, *Klebsormidium* and *Ulothrix* (Mattox and Bold, 1962; Pickett-Heaps, 1975). Furthermore, the simple filamentous genera *Raphidonema* and *Stichococcus* have been included in the Klebsormidiales on the basis of gross morphology and cell division characteristics (Mattox and Stewart, 1984), but these genera lack centrioles and flagellate life history stages and have not been included in comprehensive molecular phylogenetic analyses (Graham, 1993; Graham and Wilcox, 2000). These examples provide more evidence of thallus morphology, in this case unbranched filaments, evolving independently in phylogenetically unrelated lineages of green algae. For this reason, consideration of cytological and developmental characters is worthy of discussion

Recent molecular analyses (Karol et al., 2001; Turmel et al., 2002b) and investigation of cellular characteristics at the LM, SEM, and TEM levels (Cook, 2004) support the inclusion of two genera, *Klebsormidium* and *Entransia*, in the Klebsormidiales lineage. Although a description of isogamous sexual reproduction in *Klebsormidium* is referred to in several publications (Mattox and Bold, 1962; Pickett-

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Heaps, 1975), sex is generally not believed to occur in *Klebsormidium* (Graham and
Wilcox, 2000); comprehensive descriptions of flagellate stages in this genus are
limited to zoospores, a mode of asexual reproduction (Marchant et al., 1973; Pickett-
Heaps, 1975). Likewise, zoosporogenesis has recently been documented in *Entransia*
(Cook, 2004), refuting previous hypotheses allying *Entransia* with the Zygnematales,
a later-diverging charophyte lineage that lack centrioles and flagellate cells.

Pickett-Heaps and Marchant (1972) predicted *Klebsormidium* zoospore
ultrastructure would be more like known charophyte motile cells (*Coleochaete*
zoospores and *Chara* spermatozoids) than Ulotrichalean zoospores. This conjecture
was upheld when zoospore structure in *Klebsormidium* was documented with light
and electron micrographs (Marchant et al., 1973; Pickett-Heaps, 1975). Zoospores of
Entransia have not been directly observed (Cook, 2004), but are presumably similar
to those of *Klebsormidium* and other algal charophytes. Within the walls of the
parental vegetative cell, a single zoospore develops from the protoplast (Pickett-
Heaps, 1975). The chloroplast bulges to form a papilla in the parental cell wall,
terminal vacuoles are reduced, the microbody dissociates from the nucleus, centrioles
move to the periphery of the cell and flagella form. A contractile vacuole appears
near the basal bodies, which also develop an associated MLS. The mature zoospore
emerges, chloroplast leading and flagella trailing, from a pore created by localized
disintegration of the papilla structure; this mechanism of release is considered a
derived state relative to the general dissolution of the parental cell wall in
Chlorokybus (Graham and Wilcox, 2000). Predictably, the free zoospore is ovoid in
shape with attachment of two flagella described as lateral or subapical. Unlike

Mesostigma and motile charophyte cells, both the body and flagella of the wall-less

Klebsormidium zoospores are naked, lacking scales and hairs (Rogers et al., 1980;

Graham and Wilcox, 2000). Zoospore covering in *Entransia* is unknown, but

revelation of these characters would allow testing of the hypothesis that loss of scales

and hairs occurred shortly after the divergence of the Klebsormidiales from the

Charophyta lineage (Graham and Wilcox, 2000). Zoospores contain a single parietal

chloroplast. The plastid partially cups the nucleus, includes a single starch-

surrounded pyrenoid, and lacks an eyespot. Flagellar basal bodies are attached to a

microtubular band that extends the length of the cell and is associated with a MLS up

to several times as broad as that found in *Chlorokybus* zoospores. In contrast to

Mesostigma and *Chlorokybus*, the flagellar apparatus in *Klebsormidium* is not closely

associated with the microbody. After the zoospore swims for about an hour, flagella

are folded back along the cell and apparently absorbed directly through the membrane

and into the cytoplasm (Pickett-Heaps, 1975; Rogers et al., 1980). Flagella seem to

beat for a short period after absorption, as wave-like movements of the membrane

appear to travel around the cell from the site of flagellar attachment (Marchant et al.,

1973). Without forming a holdfast, the cell rounds up and secretes a cell wall.

Germination in *Entransia* sometimes results in the terminal cell generating a spine-

like projection and attaching to a substrate via a shapeless adhesive of unknown

composition (Cook, 2004).

A series of linear divisions of the single *Klebsormidium* vegetative cell

produce an unbranched filament, consisting of cylindrical cells generally somewhat

greater in length than diameter. Thalli of *Entransia* take the same form, but cell size

can be larger and more variable than that of *Klebsormidium*. Intercalary growth is responsible for filament elongation in both genera. Although straight filaments are most prevalent, in *Entransia* (Cook, 2004) and some species of *Klebsormidium* (Lockhorst, 1996), spiraling of the filaments—individually, in pairs, or as groups of many—can occur. *Klebsormidium* and *Entransia* cells contain a single parietal plastid that covers a minor portion of the inner cell surface. *Klebsormidium* chloroplasts are basically plate-like and contain a single, land plant-like pyrenoid with traversing thylakoids and associated starch grains. In contrast, chloroplasts of *Entransia* are fimbriate—featuring lobes, fingers, or drip-like projections—and enclose multiple pyrenoids, structurally similar to those found in *Klebsormidium* plastids. Along the plastid-free lateral wall of *Klebsormidium* lies the nucleus, with a single nucleolus. Sandwiched between the plastid and nucleus is a small microbody; close between the microbody and the chloroplast, as well as synchronized cleavage of the two organelles during cell division, is also described in *Coleochaete*. Persistent centrioles are generally found at one edge of the microbody. Terminal vacuoles are present at both ends of the *Klebsormidium* cell, but vegetative cells of *Entransia* generally feature a single large vacuole (Cook, 2004).

Cell division in *Klebsormidium* (Floyd et al., 1972; Pickett-Heaps, 1972; Pickett-Heaps, 1975) and Entransia (Cook, 2004) is generally similar to the mitotic and cytokinetic processes observed in *Chlorokybus*. Mitosis is open and centric. In preprophase *Klebsormidium* cells elongate and cleavage of the plastid and pyrenoid begins. With prophase, cortical microtubule arrays disperse and centrioles replicate and migrate, establishing the poles of the forthcoming spindle. As the spindle forms

both the nucleus and microbody elongate drastically. By metaphase the microbody is

split in two and disruption of the nuclear envelope is complete. In the anaphase

interzone two small vacuoles appear and then fuse into a single vacuole that expands

as the original terminal vacuoles shrink. The distance from chromosome to spindle

pole is constant as the interzone expands. Some evidence suggests that a single

vacuolar network is formed by contiguous terminal and interzonal vacuoles (Pickett-

Heaps, 1975). Interzonal microtubules persist throughout telophase and cytokinesis,

maintaining a great distance between the two daughter nuclei. Cytokinesis initiates as

a diaphragm-like invagination of the cell membrane, first appearing during

metaphase, contracts, cutting the interzonal vacuole into two terminal vacuoles, one

for each of the newly formed daughter cells. Cross-walls, often with lateral

extensions that form an H-shape, are secreted. The arrangement of organelles in each

daughter cell returns to interphase conditions—microbody and centrioles between the

nucleus and chloroplast with terminal vacuoles capping each end of this complex.

Irregularities and protuberances, or even interruptions through which the nucleus can

pass from one cell to an adjoining cell, have been observed in the central cross wall of

some *Entransia* cells (Cook, 2004). How these structures form is not clear, but Cook

(2004) has hypothesized a possible evolutionary connection between these structures

and potential innovations related to the origin of phragmoplastic cytokinesis.

Entransia cells, as well as those of *Klebsormidium*, lack plasmodesmata.

     In addition to propagation through zoosporogenesis, *Klebsormidium*

(Lockhorst, 1996) and *Entransia* (Cook, 2004) have both been reported to produce

non-motile aplanospores. Immature aplanospores have the developmental potential to

become zoospores, but, instead of forming flagella, secrete a second cell wall within the walls of the parental vegetative cell. Germination initiates while aplanospores are still associated with the vegetative thallus and results in typical filaments of cylindrical cells. Furthermore, both genera are also capable of propagation through fragmentation of the vegetative thallus. *Klebsormidium* and *Entransia* are characterized by interlocking, H-shaped cell wall segments that can pull apart, dividing a single filament into two or more smaller thalli with collar-like termini. In *Entransia* very short dead cells seem to be specialized for facilitation of thallus fragmentation (Cook, 2004). It is currently unknown whether or not this phenomenon is homologous to other instances of programmed cell death, a mechanism important to the morphogenesis of numerous algal thalli and land plant bodies.

2.4   Zygnematales

Relative to earlier-diverging charophyte lineages, the Zygnematales are a morphologically diverse group comprising at least a thousand described species. Traditional classification schemes of the Zygnematales define three distinct groups. The family Zygnemataceae (*sensu* Bold and Wynne, 1985) includes unbranched filaments. Some examples are members of the genera *Mougeotia*, *Spirogyra*and *Zygnema* (for which the order and family are named). The thalli of these algae are morphologically similar to *Klebsormidium* and *Entransia*, except that they do not have collar-like H-shaped cell walls and the corresponding tendency for frequent filament fragmentation. Also distinguishing the Zygnemataceae from the algae of the Klebsormidiales, some filamentous members of the Zygnematales feature terminal

cells that serve as holdfasts and develop into elaborate lobed shapes. In fact, a diverse collection of intricately shaped cells characterizes both filamentous and single-celled thalli of zygnematalean algae. Saccoderm desmids, algae of the Mesotaeniaceae (*sensu* Bold and Wynne, 1985), are unicells with pore-less walls of homogeneous composition. Genera comprising this family include *Cylindrocystis*, *Mesotaenium*, *Netrium*, and *Spirotaenia*. Algae classically placed in the two families Zygnemataceae and Mesotaeniaceae possess chloroplasts with a wide range of morphologies, from plate-like parietal to highly lobed or fimbriate, and even spiraling, as in the case of the familiar *Spirogyra* Analyses of DNA sequence data (McCourt et al., 1995) indicate that organismal phylogeny may be more accurately reconstructed based on plastid form than thallus complexity. McCourt et al. (1995) demonstrated that unicellular and filamentous algae sharing similar plastids group together to form monophyletic groups (McCourt et al., 1995). Such results demand reevaluation of the Zygnemataceae and Mesotaeniaceae, as this study suggests the two families are not natural groups. The Desmidiaceae, or placoderm desmids, are algae that feature unique morphologies. Defining this group are two characters— thalli constructed of two semicells connected by a thin isthmus, and a specialized cell wall with distinctive pores. Representative organisms include unicells—the highly ornate *Micrasterias* and *Staurastrum*, as well as the simpler *Closterium* and *Cosmarium*—and pseudofilaments of loosely connected cells, like *Desmidium*. Placoderm desmids often possess chloroplasts that are similar to cell shape. So far, taxonomic and phylogenetic hypotheses that incorporate this three-family system of classification have not stood up well to the rigorous testing of molecular analyses

(McCourt et al., 1995; 2000; Karol et al., 2001). Additional work is needed to resolve the phylogenetic relationships among algae of this very large and complex order. Such studies are currently underway (John Hall, personal communication).

Despite the tremendous diversity of the Zygnematales, members of this lineage have several unique characteristics in common (Graham and Wilcox, 2000). Centrioles and flagellate life history stages are absent in all known members of the Zygnematales. This lineage is also characterized by a specialized mode of sexual reproduction, conjugation. Cell shapes, chloroplast structure, and zygote morphology vary greatly across the algae of the Zygnematales. The overwhelming diversity, lack of flagellate cells, and sex via conjugation distinguish the algae of this lineage from other charophytes. Yet, analyses of morphology, ultrastructure, biochemistry, and molecular sequence data have all confirmed this monophyletic clade of green algae diverged from within the Charophyta lineage.

Even with great variation in cell and thallus morphology, algae of the Zygnematales share a number of cytological features, both among members of the order and with other charophytes. Cells are generally mononucleate and possess peroxisomes (microbodies) typical of other algal charophytes and land plants. Chloroplasts, although variable in shape and size, contain one or many pyrenoids, similar to those known in other lineages of the Charophyta.

Primary walls of cells in filamentous forms and related saccoderm desmids contain both cellulose and other carbohydrates in a combination similar to that found in the genus *Klebsormidium* (Hotchkiss et al., 1989). These cells may also feature a thick secondary wall and a layer of extracellular matrix material, often composed of

hemicelluloses and calcium pectate. Placoderm desmids develop a secondary wall and distinctive outer layer, both of which commonly feature complex patterns of bumps, ridges, spines, and other ornamentations. The primary wall of these cells is often lost during the development of the secondary wall. Alternatively, bits of primary wall may remain in pseudofilamentous placoderm desmids. This retained material may play a role in holding individual cells together in long, linear arrays (Krupp and Lang, 1985). A pectin-rich mucilage is released from pores in the secondary walls of placoderm species. This process of mucilage extrusion has been hypothesized as a mechanism that explains a gliding-type of cell motility observed in some desmids (Hader and Hoiczyk, 1992).

Plasmodesmata have not been reported in algae of the Zygnematales. Cytokinesis in some filamentous members of the Zygnematales is thought to incorporate a combination of typical centripetal furrowing and centrifugal development involving a unique structure that resembles a small phragmoplast (Graham, 1993; Graham and Wilcox, 2000). This unique process has been proposed as a possible intermediate state in the evolutionary transition between furrowing cell division, typical in *Chlorokybus* and *Klebsormidium*, and phragmoplastic cytokinesis, present in land plants and the algal orders Coleochaetales and Charales.

Lacking zoospores, algae of the Zygnematales can reproduce asexually through fragmentation of filaments, mitotic and cytokinetic division of unicells, or production of dormant cells—aplanospores, akinetes, or parthenospores—that feature specialized walls containing resistant compounds. Without flagellate isogametes or motile sperm cells, members of this order undergo sexual reproduction by the process

of conjugation. Conjugation involves the pairing of individual unicells or filaments, often resulting in two thalli sharing a common mucilaginous enclosure, and a physical connection through which protoplast-derived gametes meet and subsequently fuse and then develop into ornate and morphologically diverse zygotes with resistant walls (Bold and Wynne, 1985). Although flagellate cells are not present in algae of the Zygnematales, the process of development from single cell to multicellular thallus occurs in the process of germination of specialized dormant cells and zygotes.

2.5   Coleochaetales

The order Coleochaetales is named for the genus *Coleochaete*, algal charophytes that exhibit a diverse array of thallus morphologies and cytological characters often hypothesized to be homologous to the specialized features of land plants. *Coleochaete* possesses distinct seta cells, characterized by sheathed hairs thought to be unique to this lineage of green algae. Based on similarities in thallus and zoospore morphology, and especially due to the presence of sheathed hairs, the genus *Chaetosphaeridium* is also likely a member of the order Coleochaetales. This relationship is currently somewhat controversial, as some molecular analyses have resulted in phylogenies that support this hypothesis while other studies suggest alternative placements of *Chaetosphaeridium*. Other genera bearing morphological and cytological resemblance to *Coleochaete* have not yet been included in molecular phylogenetic examinations of this order.

Asexual reproduction in *Coleochaete* occurs via zoospore production and propagation. Features of zoospores (Sluiman, 1983) are similar to flagellate cells

found in other algal charophytes; they are wall-less but scaly cells, spherical to ovoid in shape, possessing two flagella inserted subapically or laterally, a single chloroplast with a starch-coated pyrenoid but lacking an eyespot, and a flagellar apparatus including connecting fiber-linked basal bodies and two root structures, one composed of few microtubules and a second broader root that incorporates a single MLS. Zoospores of *Chaetosphaeridium* are also characterized by these features (Moestrup, 1974).

Graham and McBride (1979) investigated the spermatozoids of the discoid species *Coleochaete scutata* and found that they are more specialized than, but also similar to *Coleochaete* zoospores. About one-quarter the size of zoospores, spermatozoids are scaly biflagellates, spherical to ovoid in shape. Relative to zoospores, both the contents of the nucleus and cytoplasm in spermatozoids have increased density and decreased volume. The spherical nucleus inhabits most of the central region of the spermatozoid with a portion extending to the flagellar apparatus in the apex of the cell. Clusters of small mitochondria, potentially homologous to the large mitochondrion found in land plant male gametes, are also found near the basal bodies in the anterior portion of the specialized sex cell. In the posterior region of the spermatozoid lies a single plastid, reduced and packed with large starch granules, much like the amyloplasts found in spermatozoids of charalean algae and bryophytes. Spermatozoids cells are wall-less, covered with flat body scales, unlike the three-dimensional conical or pyramid-shaped scales of *Coleochaete* zoospores. Subapically or laterally inserted flagella are covered with hairs and flat, polygonal scales. A flagellar apparatus root structure is composed of a band of microtubules that extends

along the periphery of the cell. At the anterior end of the cell, close to the flagellar basal bodies, the microtubular band terminates with an MLS, smaller than but similar in organization to those found in *Coleochaete* zoospores. Graham and McBride (1979) compare the morphology and cytological organization of *Coleochaete* spermatozoids to those of *Lycopodium*, a genus of land plants generally categorized as fern allies. Although the round spermatozoid differs from the elongate, helical-shaped flagellate sex cells of charalean algae and bryophytes, Delwiche et al. (2002) have observed the elongation of *Coleochaete* spermatozoids as they approach sessile egg cells.

In *Coleochaete* sexual reproduction is oogamous. Both homothallic and heterothallic species of *Coleochaete* have been described (Graham and Wilcox, 2000). Sperm are produced in specialized antheridial cells. In some species antheridia form small extensions of translucent cells that develop in close proximity to egg cells (Graham and Wedemayer, 1984); other species feature antheridia development by asymmetric divisions of cells grouped in concentric rings halfway between the center and perimeter of the discoid, parenchymatous thallus (Graham, 1993). Egg cells are retained by the *Coleochaete* thallus and are characterized by a cell wall protuberance, the trichogyne. Both antheridia and trichogynes exhibit localized cell wall disintegration, to allow spermatozoid release and fertilization of the egg, respectively. Zygotes are retained by the parental thallus, and in some species corticating vegetative cells grow around or completely surround the developing zygote. Such corticating cells sometimes feature intricate cell wall ingrowths (Graham and Wilcox, 1983), hypothesized to facilitate the flow of nutrients

from vegetative cells to zygotes. Graham et al. (1991) speculated that corticating

cells with wall ingrowths in *Coleochaete* might be homologous to the placental

transfer cells found in embryophytes. Zygotes enlarge due to accumulation of

nutrients. Both zygotes and corticating cells are known to feature linings composed

of resistant materials similar to protective compounds found in land plants (Delwiche

et al., 1989). Zygotic meiosis and a series of subsequent mitotic divisions (Hopkins

and McBride, 1976) result in the release of haploid meiospores, scaly biflagellates

that develop flagellar apparatus featuring an MLS root and other characteristics

similar to *Mesostigma* and the charophyte motile cells described above (Graham,

1993; Graham and Wilcox, 2000). Meiospores swim, settle, and develop into new

vegetative thalli. Sexual reproduction in *Chaetosphaeridium* has also been described

(Thompson, 1969), but the phenomenon has not been documented photographically.

Development of the *Coleochaete* vegetative thalli from single-celled

zoospores or meiospores proceeds via apical growth, as all morphological variants of

the genus feature terminal or marginal meristem cells (Graham, 1993). After

flagellate cells cease motion, the cells become round, settle, and attach to the

substrate. A transverse cell division creates an asymmetrical stack of two cells. The

smaller upper cell differentiates into a seta cell by developing a sheathed hair. The

lower cell begins a series of divisions, variable in pattern across the species of

Coleochaete (Mathew Cimino, personal communication), resulting in development of

the vegetative thallus.

Various thallus morphologies are found in the genera *Coleochaete* and

Chaetosphaeridium, but all are examples of branched filaments with apical or

peripheral growth (Graham, 1993; Graham and Wilcox, 2000). Several *Coleochaete*

species, as defined in recent molecular analyses (Delwiche et al., 2002), possess

prostrate, branched filaments that form by Y-shaped cell divisions. In such cell

divisions, branching occurs when a vegetative cell develops a protuberance that

elongates before it is eventually divided from the parental cell by cytokinesis. Other

forms include prostrate and erect filaments, both featuring Y-shaped cell division.

While most species generally grow into circular or hemispherical forms, some are

specialized for epiphytic growth, following the pattern of cell boundaries on aquatic

embryophyte substrates, or endophytic growth, between the cell walls of fellow algal

charophyte *Nitella* (Cimino and Delwiche, 2002). Distances separating adjacent

filaments in round thallus forms also vary from species to species. Due to very

tightly packed arrangements of branched filaments, forms featuring Y-shaped

division may misleadingly appear to be discoid, "parenchymatous" thalli; such forms

are considered pseudoparenchymatous (Graham, 1993). Alternatively, when

filaments are extremely tightly arranged due to a series of circumferential and radial

divisions (cytokinesis occurring in two perpendicular planes), the thallus takes on a

true disc-like appearance. Discoid species featuring these perpendicular cell divisions

(T-shaped cell division *sensu* Delwiche et al., 2002) exhibit a growth pattern

reminiscent of parenchymatous tissue organization within the embryophytes

(Graham, 1993). Although this parenchymatous organization appears in tissues of the

Coleochaetales, Charales, and land plants—presumably the three clades composing

the monophyletic group at the apex of the Charophyta lineage—because parenchyma

is not found in the more basal members of the Coleochaetales, and is not known to

have been lost by these less-derived algae, parenchyma in *Coleochaete* is not homologous to parenchyma found in the Charales and land plants. Also similar to cells of the Charales and land plants, cross walls in discoid species of *Coleochaete* are developed via cytokinesis featuring a phragmoplast (Brown et al., 1994). Plasmodesmata have also been described in discoid thallus forms (Graham, 1993; Graham and Wilcox, 2000). Although documentation has not yet been published, observations of phragmoplastic cytokinesis and plasmodesmata have been made in non-discoidal forms of *Coleochaete* and *Chaetosphaeridium* (Martha Cook, personal communication). If further studies confirm these observations, homology of phragmoplasts and plasmodesmata found in the Coleochaetales, Charales, and embryophytes would appear a likely hypothesis. Only several percent of *Coleochaete* cells develop sheathed hairs; generally every cell of the *Chaetosphaeridium* thallus generates this distinctive structure.

2.6   Charales

Genera comprising the living members of the order Charales are *Chara*, *Lamprothamnium*, *Lycnothamnus*, *Nitella*, *Nitellopsis*, and *Tolypella*. Additionally, a vast record of charalean fossils has been described. This lineage includes many more extinct than extant forms, a potentially problematic cause of sampling error that may affect attempts to construct phylogenetic hypotheses from molecular data (McCourt et al, 1996).

Members of the Charales are considered to be among the most complex algae in terms of vegetative morphology, reproductive systems, and developmental

processes (Graham, 1993; Graham and Wilcox, 2000). Thalli of some species may attain lengths of well over one meter. Vegetative morphology is organized into a type of nodal arrangement. Both the tremendous size and nodal organization of the charalean thallus may cause some observers to confuse these algae with aquatic embryophytes. However, there are several important differences between morphology in algae of the Charales and the bodies of land plants. Nodal organization in the Charales is a feature of the haploid (gametophyte) thallus, yet it is generally, to the exception of mosses and leafy liverworts, the diploid sporophyte generation of land plants that possesses a nodal arrangement. Furthermore, in the Charales the "nodes," "internodes," and "branches" of the thallus are each composed single celled sections. Generally, each component of the nodal structure in embryophytes is multicellular.

Algae of the Charales are fundamentally quite similar to those of the Coleochaetales in that thallus forms in both orders are essentially branched filaments of cells, some of which may differentiate to perform specialized functions. Many times larger than those of the Coleochaetales, charalean thalli also feature a more complex body that can be described as several distinct regions distributed along a main axis of cells.

Erect portions of the charalean thallus, superficially resembling the shoot system of land plant bodies, extend out of and above the sandy substrate of benthic habitats. The single, most distal cell of the "shoot" portion is specialized to function as an apical meristem. In contrast to the tissue-producing meristematic cells of land plants, which have multiple cutting faces, the apical cell in algae of the Charales

divides only transversely from the lower cutting surface. Division of the meristem produces a derivative directly below the apical cell. This cell is commonly called a segment cell.

The remainder of the charalean shoot develops from the segment cell (Cook et al., 1998). First, the segment cell divides transversely, resulting in a stack of two cells, each shaped like a disc or short cylinder. The upper of the two cells develops into a node cell; the lower cell becomes the internode.

In development of the node complex, initial nodal cells first divide in half longitudinally, creating two adjacent semicircular node cells, as viewed in cross-section. These two cells then alternate turns of directed, asymmetrical cell divisions that give rise to a peripheral whorl of small cells, called branch initials. The coordinated division of branch initials has been compared to the development of antheridial initials in discoid species of *Coleochaete* (Graham 1996; Graham and Wilcox, 2000). Like the apical cell of the main axis, branch initials of the nodal complex serve as meristematic cells that generate lateral branches. Branches of the thallus are also organized into a nodal configuration, similar in structure and development to the main axis, with alternating internode and node cells, of which the latter may generate smaller branchlets.

An internode cell develops from the lower derivative of the event dividing the segment cell transversely. Internode cells grow from disc-like into extremely elongate cylindrical cells. Single internode cells may attain lengths between 10 and 20 cm. In species of the genus *Chara*, the outer surface of internode cells may be

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covered by a layer of corticating cells. Corticating filaments, columnar lines of cells, grow from the nodal complexes at both termini of the long internode cell.

The apical cell and alternating system of nodal complexes (node cells, branches, branchlets) and internode cells make up the erect, shoot-like portion of typical charalean thalli. Attached to solid substrates or beneath the level of penetrable surfaces is the most basal part of the thallus, a system of rhizoids. Rhizoids are very long, colorless cells that elongate via tip growth. Cell polarity and distinct, specialized zones within the cells of *Chara* rhizoids have been described (Kiss and Staehelin, 1993). Functionality of such zones—including vacuole, nuclear, plastid, clear, statolith, and apical—is not well understood and requires more investigation. Modes of geotropism and asexual reproduction may be related to components of the rhizoid.

Zoospores are not produced by algae of the Charales, but other variations of asexual reproduction have been reported. New thalli may be generated directly from nodal complexes, rhizoids, or severed parts of existing thalli. Also, some species of the Charales may produce bulbils on rhizoidal surfaces. Bulbils can be spherical or ovoid in shape, although sometimes are more ornate, and are generally light in color. These structures can develop into new thalli and presumably serve as a dispersal mechanism.

A variety of interesting cytological characters are present in the different types of cells that make up thalli of the Charales. During cell division in vegetative cells, mitosis is open, a trait typical of charophytes. Unlike the centric mitosis described in other algal members of the lineage, however, cells of charalean algae lack centrioles

at the poles of mitotic spindles. Cytokinesis in the Charales involves a land plant-like phragmoplast (Pickett-Heaps, 1975; Cook et al., 1997). Plasmodesmata are also present in cells of the Charales, although these structures have been both compared and contrasted to the plasmodesmata found in land plants (Cook et al., 1997). Internodal cells are multinucleate, containing as many as several thousand nuclei. Plastids in rhizoidal cells evidently lack pigment and function in the storage of starch, like amyloplasts in land plants.

Sexual reproduction in the Charales is oogamous and characterized by complex gametangia that feature protective coverings of corticating vegetative cells. Both male and female gametangia form at nodal cells of branchlets. Antheridia develop according to a complex program of divisions that results in sperm-producing reproductive cells enclosed by an outer layer of shield cells, arranged in groups of eight (Pickett-Heaps, 1975). From internal reproductive cells, unbranched filaments of tiny cells develop. Each sperm filament cell can produce a single, helically twisted spermatozoid, and mature spermatozoids are released by the separation of shield cells. Development of oogonia commences as an initial cell undergoes two consecutive transverse divisions; the upper cell becomes the egg, the lower cell is designated a basal stalk cell, and the middle cell undergoes a series of divisions that generates five peripheral tube cells (Pickett-Heaps, 1975). As the egg cell enlarges, tube cells elongate and extend around it in a counter-clockwise helical pattern, essentially corticating and protecting the precious sex cell. The tip of each tube cell, upon reaching the apex of the egg, divides transversely once or twice, producing a crown of coronal cells. When oogonia mature, gaps form between tube cells in order

to allow fertilization to occur. Zygotes form thick walls that incorporate resistant

materials. Zygotes are retained on the parental thallus until maturity, at which time

they may separate and disperse. Members of the Charales are thought to undergo

zygotic meiosis, although this is based on indirect observations (Graham and Wilcox,

2000). Germination of a zygote occurs when the protonema, a translucent filament,

emerges from a crack in the zygote wall. A new vegetative thallus develops from the

protonema, initiating the beginning of a new life history cycle.

Chapter 3: Testing Phylogenetic Hypotheses

3.1   Introduction

Understanding evolutionary transitions between lineages of the algal Charophyta is made possible when structural and developmental characters can be mapped onto trees representing hypothesized phylogenetic relationships. Using ultrastructural, morphological, and developmental characters to generate hypotheses of phylogeny (such as in Graham et al., 1991) is a worthwhile exercise in that it facilitates consideration of many possible transition series hypotheses. However, mapping and analyzing structure and development onto trees that have been reconstructed from structural and developmental characters is a circular and thus uninformative, tautological method of investigating evolutionary trends (Raff, 1996). Ideally, characters independent of structure and development should be utilized to build phylogenies intended for use in polarizing transition series of morphological and developmental character states. The advent of molecular methods has resulted in vast amounts of DNA sequence data that can be used as characters, independent of structure and development, in phylogenetic studies. Large datasets composed of collections of aligned, homologous DNA base pairs can be analyzed with systematic methods that test many different phylogenetic hypotheses objectively, and even probabilistically. Furthermore, knowledge of genome architectural character states— variations in how DNA markers are arranged within a genome—can provide another type of data, independent of physical structure and development, for testing alternative hypotheses of organismal evolutionary relationships.

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Recent phylogenetic analyses of DNA sequence data (Bhattacharya et al., 1998; Karol et al., 2001; Turmel et al., 2002b) have supported a monophyletic clade, the Charophyta, that includes land plants and the green algal orders discussed above. These studies have also generally supported the same order of divergence for the lineages of the Charophyta. Current understanding of the phylogenetic relationships between orders of the Charophyta indicate a likely sequence of evolutionary branching to be, from earliest- to latest-diverging, the Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales, Charales, and embryophytes. Although these features of Charophyta phylogeny are generally congruent among analyses of various types of data, the phylogenetic placement of several genera remains controversial.

Mesostigma, the motile, scaly, biflagellate unicell is one such enigmatic taxon. Analysis of actin gene sequence data (Bhattacharya et al., 1998) and the four-gene analyses of Karol et al. (2001) both support the placement of *Mesostigma* within the Charophyta clade as the earliest-diverging lineage among taxa sampled. Analyses of characters from sequenced chloroplast (Lemieux et al., 2000) and mitochondrial genomes (Turmel et al., 2002a) support an alternative placement of *Mesostigma* at the base of the Viridiplantae prior to the divergence between the two major green lineages, Chlorophyta and Charophyta. Furthermore, based on DNA sequence analysis of nuclear ribosomal RNA small subunit (SSU, 18S) data, Marin and Melkonian (1999) proposed the naming of a new algal class, the Mesostigmatophyceae, including *Mesostigma* and *Chaetosphaeridium* as sister taxa.

Traditionally, morphological characters—possession of *Coleochaete*-like sheathed hairs and a branched filamentous thallus—have supported a sister relationship between *Coleochaete* and *Chaetosphaeridium* within the order Coleochaetales (Graham et al., 1991; Graham, 1993; Graham and Wilcox, 2000). Despite this, other analyses of nuclear ribosomal RNA SSU gene data (Sluiman and Guihal, 1999; Cimino et al., 2000; Sluiman, 2000) support the inclusion of *Chaetosphaeridium* at the base of the Charophyta clade, and not allied with *Coleochaete*.

Before phylogenetic analysis of DNA characters became commonplace, Manhart and Palmer (1990) presented one of the first bits of molecular evidence supporting the monophyletic alliance of land plants and members of the green algal orders Zygnematales, Coleochaetales, and Charales. The transcribed spacer region is the segment of chloroplast genomes typically flanked by the genes coding for chloroplast ribosomal RNA small- (16S) and large-subunits (23S). Within this region, nearly all land plants sampled were known to have two chloroplast transfer RNA (tRNA) genes, tRNA-Ala and tRNA-Ile, interrupted by introns. On the phylogeny of primary plastids, organisms basal to the Viridiplantae—Rhodophyta, Glaucocystophyta, and cyanobacteria—lack introns within chloroplast tRNA-Ala and tRNA-Ile genes. Looking for introns, homologous to those known in land plants, in the algal charophytes, Manhart and Palmer (1990) isolated and sequenced portions of the transcribed spacer region containing the two tRNA genes from *Spirogyra maxima* (Zygnematales), *Coleochaete orbicularis* (Coleochaetales), and *Nitella axillaris* (Charales). The results of this study support a monophyletic group composed of

charophyte green algae and the land plants, with the Charales and Coleochaetales sharing the closest relationships with embryophytes.

Here, using the genome architectural characters explored by Manhart and Palmer (1990), hypothesized relationships involving the genera *Mesostigma* and *Chaetosphaeridium* have been tested. Furthermore, due to the confounding results of previous analyses of charophyte nuclear rRNA SSU genes, a different nuclear gene, encoding the actin protein, was characterized from strains of *Chaetosphaeridium* and *Coleochaete*. Sequences from these taxa were not included in previous analyses of charophyte actin genes (Bhattacharya, 1998; An et al., 1999). Analyses of chloroplast genome architectural data and nuclear protein coding DNA characters have shed additional light on the phylogenetic placement of the genera *Mesostigma* and *Chaetosphaeridium*.

3.2   Materials and Methods

Algal strains used in this study were obtained from UTEX, SAG, and Delwiche Lab (CFD) culture collections. Cultures were grown in liquid medium, Guillard's Woods Hole MBL or DY III. Cycles of 16 hours light and 8 hours dark at a temperature of 20°C were controlled within a culture chamber. DNA was isolated using Nucleon Phytopure kits from Amersham Biological. Manufacturer's protocol was modified by inclusion of a second chloroform extraction in order to further eliminate contaminating cellular debris and mucilage. Amplification of chloroplast transcribed spacer regions and actin genes was achieved through the use of polymerase chain reaction. DNA primers were based on published primer sequences

(Bhattacharya et al., 1998) or were designed by investigator Lewandowski using Amplify v. 1.2 software. DNA sequencing reactions were completed using Big Dye terminator v. 2.0 (ABI Technology) kits. Reactions were read on ABI 3100 capillary systems. Sequence data was compiled with Sequencher v. 3.1 software (GeneCodes). Alignments were manipulated using MacClade. Distance matrices for estimation of intron sequence similarity were estimated using the software package PAUP* (Swofford, 2001).

Phylogenetic analyses of the actin dataset were conducted in PAUP* (Swofford, 2001). Optimality criterion was set to maximum likelihood using the general time reversible nucleotide substitution model (GTR+I+Γ). A full heuristic search was conducted with tree bisection and reconnection (TBR) branch swapping. Bootstrapping of the dataset (203 replicates) was conducted using the nearest neighbor interchange (NNI) type of branch swapping. Additional chloroplast transcribed spacer region and nuclear actin gene sequences were obtained from GenBank (NCBI).
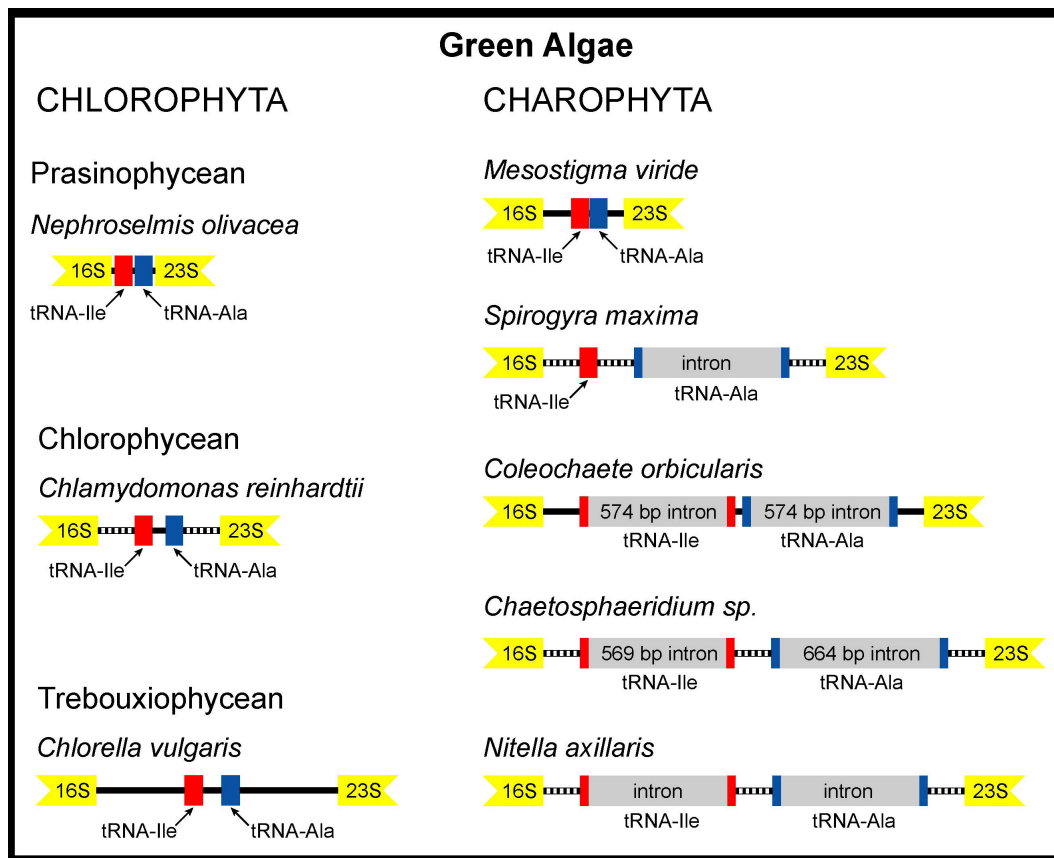
3.3   Results

3.3.1   Chloroplast Transfer RNA Gene Introns
Amplification and sequencing of the transcribed spacer region of chloroplast DNA isolated from several algal charophyte genera has revealed the following results.

Both the tRNA-Ala and tRNA-Ile chloroplast genes of *Mesostigma viride* were found to be continuous, uninterrupted by introns (Fig. 1). This finding was

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Figure 1. Illustrations of the structure of chloroplast transcribed spacer regions in various examples of green algae from the two major lineages of the Viridiplantae, the Chlorophyta and Charophyta. Data for *Chaetosphaeridium* were generated in this study, and data for *Mesostigma* and *Coleochaete* were collected in order to confirm previously published results. Illustrations representing chloroplast genome structure in other pictured genera were drawn from existing data, available in GenBank (NCBI). Furthermore, the chloroplast transcribed spacer region was also sequenced for *Chlorokybus atmophyticus* (UTEX); both genes, for tRNA-Ala and tRNA-Ile, were found to be continuous, not interrupted by introns (not pictured).

corroborated by the publication of the complete chloroplast genome of *Mesostigma* (Lemieux et al., 2000).

Confirming the results of Manhart and Palmer (1990), both tRNA-Ala and tRNA-Ile chloroplast genes of *Coleochaete orbicularis* (UTEX 2651) were found to contain introns (Fig. 1). Some differences between the DNA sequences produced by Manhart and Palmer (1990) and those of this study were observed and are possibly explained by errors frequently encountered in the manual sequencing methods utilized by Manhart and Palmer (1990). Yet, the base composition of both gene and intron sequences determined by this author are fundamentally similar to those previously published by Manhart and Palmer (1990). Intron insertion position was the same in sequences determined by both studies. For both the tRNA-Ala and tRNA-Ile genes, intron length in *Coleochaete orbicularis* is approximately 570 base pairs. Variation in PCR product length, possibly due to template DNA secondary structures, has made for difficulty in determining exact intron lengths.

In two distinct strains of the genus *Chaetosphaeridium*, SAG 26.98 and CFD 5c1, chloroplast genes for tRNA-Ala and tRNA-Ile were both found to be interrupted by introns (Fig. 1). The intron contained in the gene for tRNA-Ala is approximately 660 base pairs in length. Sequence similarity (60-75%, depending on alignment) between this intron and the intron found in tRNA-Ala of *Coleochaete* is the basis for hypothesizing homology between these two introns. Insertion position within the tRNA-Ala gene is identical in *Coleochaete* and both strains of *Chaetosphaeridium*. Compared to the intron found in the tRNA-Ile gene of *Coleochaete*, the tRNA-Ile intron found in both *Chaetosphaeridium* strains is similar in length (approximately

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570 base pairs) and sequence (70-80%, depending on alignment), and is identical in position of insertion.

*3.3.2   Actin Gene Sequence Analysis*
      Four new DNA sequences were determined for the actin gene of algal charophyte strains, *Chaetosphaeridium globosum* (SAG 26.98), *Chaetosphaeridium* sp. (CFD 1c11), *Chaetosphaeridium* sp. (CFD 5c1), and *Coleochaete soluta* (CFD 32d1). The new sequences were combined with previously existing data to form a 775-base pair dataset, including 22 Viridiplantae representatives and one outgroup taxon. Heuristic search with tree bisection and reconnection branch-swapping identified a best tree with likelihood –ln L = 9100.4149. This phylogeny (Fig. 2) supports the monophyly of the Coleochaetales (54% bootstrap support), including the three *Chaetosphaeridium* and two *Coleochaete* samples. Placement of *Mesostigma* within the Charophyta clade, to the exclusion of Chlorophyta taxa and *Cyanophora*, was also supported (91%). Monophyly of algal and land plant charophyte taxa (not including *Mesostigma*) was also supported (76% bootstrap) by this phylogenetic analysis.

3.4   Discussion

3.4.1   Phylogenetic Position of Scaly Biflagellate Mesostigma
      The lack of introns in *Mesostigma* chloroplast tRNA genes supports either of two hypotheses; both placements, *Mesostigma* diverging at the base of the Charophyta or at the base of the Viridiplantae, are congruent with this genome architectural data. These data do not favor one hypothesis over the other, but are

Figure 2. Hypothesis describing phylogenetic relationships among the Viridiplantae, based on maximum likelihood analysis of a 775-base pair alignment of actin gene DNA sequences. Sequences represent land plants, algal charophytes, algae of the Chlorophyta, the enigmatic *Mesostigma*, and an outgroup taxon, *Cyanophora* (Glaucocystophyta). Numbers at horizontal branches represent bootstrap support generated by 203 maximum likelihood search replicates utilizing NNI branch swapping.
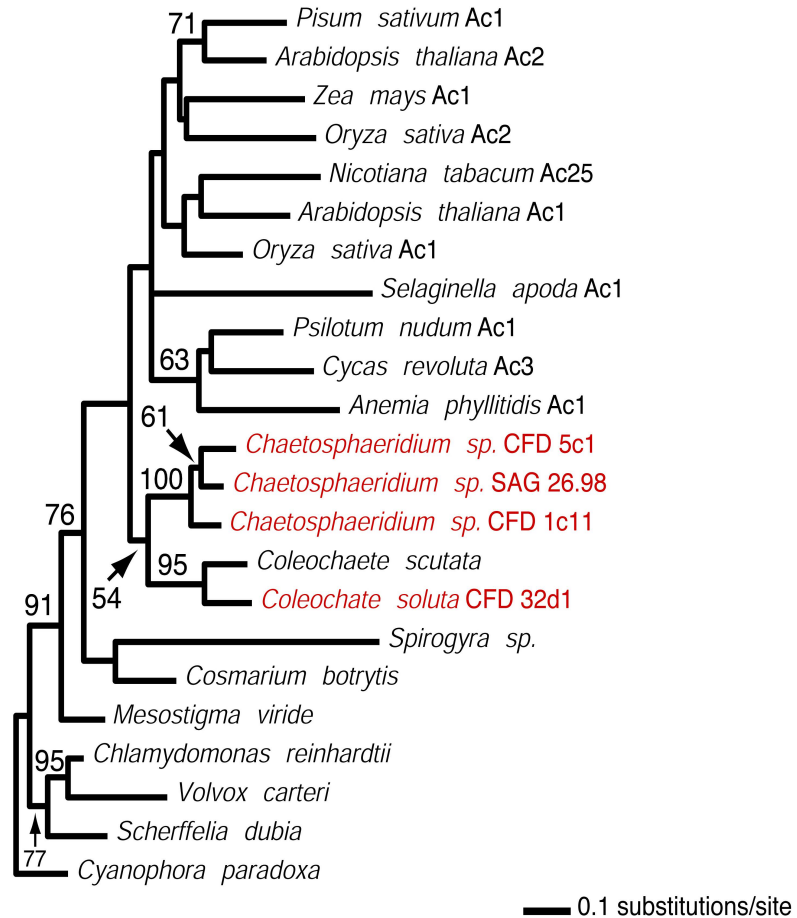
also inconsistent with other hypotheses, such as those allying *Mesostigma* with more morphologically complex algal charophytes, such as *Chaetosphaeridium*.

Likewise, the phylogeny of nuclear actin genes in the Charophyta supports the hypothesis including *Mesostigma* within the Charophyta (91% bootstrap) at the base of the lineage. Previous analysis of *Mesostigma* actin gene sequence (Bhattacharya et al., 1998) did not include *Chaetosphaeridium* in the range of taxa sampled. In this analysis, the partitioning of *Mesostigma* from *Chaetosphaeridium* and the other morphologically complex algal charophytes is supported by 76% bootstrap support. These data are not consistent with the hypothesis and taxonomic scheme proposed by Marin and Melkonian (1999). However, due to short branch lengths and incomplete taxon-sampling in this study, alternative placements of *Mesostigma* outside the Charophyte clade cannot be ruled out entirely.

3.4.2   *On the Monophyly of the Coleochaetales*
The discovery of introns in both *Coleochaete* and *Chaetosphaeridium* chloroplast tRNA-Ala and tRNA-Ile genes supports a monophyletic order, Coleochaetales that includes the genera *Coleochaete* plus *Chaetosphaeridium*. These results were later independently generated and published by another group of investigators (Turmel et al., 2002b; Turmel et al., 2002c). It may be argued that introns in *Coleochaete* chloroplast tRNA genes are homologous to those of *Chaetosphaeridium*; this hypothesis is supported by high levels of sequence similarity (greater than 70%) and identical intron insertion positions within the two chloroplast tRNA genes.

Phylogeny of charophyte actin genes (Fig. 2) also supports a monophyletic Coleochaetales, with 54% bootstrap uniting the order consisting of *Coleochaete* plus *Chaetosphaeridium*. The addition of three new *Chaetosphaeridium* taxa and a new *Coleochaete* strain distinguishes this study from prior work by Bhattacharya et al. (1998). The addition of *Chaetosphaeridium* to the actin phylogeny allows for contrasting of this nuclear gene analysis against that of the nuclear rRNA SSU sequence data analyzed by Marin and Melkonian (1999).

Phylogenetic analysis of actin gene sequence data including the three genera *Mesostigma*, *Coleochaete*, and *Chaetosphaeridium* supports a monophyletic Coleochaetales clade composed of *Coleochaete* plus *Chaetosphaeridium*. Furthermore, this analysis supports phylogenetic placement of *Mesostigma* at the base of the Charophyta clade, contrary to the alliance of *Mesostigma* and *Chaetosphaeridium* suggested by the results of Marin and Melkonian (1999). Basal placement of *Mesostigma* and a monophyletic Coleochaetales is a phylogenetic hypothesis that is also bolstered by the finding of *Coleochaete*-like introns within the chloroplast tRNA genes of *Chaetosphaeridium* (Lewandowski, unpublished results; Turmel et al., 2002b; Turmel et al., 2002c). More recent phylogenetic analyses of chloroplast, mitochondrial and nuclear ribosomal DNA sequence data also support the monophyly of the Coleochaetales (Karol et al., 2001; Turmel et al., 2002b; Delwiche et al., 2002) and the basal placement of *Mesostigma* (Karol et al., 2001; Turmel et al., 2002a).

Testing of enigmatic phylogenetic hypotheses is necessary if structural and developmental character states are to be polarized and analyzed. Both chloroplast

genome architecture characters and nuclear actin gene base pair characters are very likely to be independent from most structural and developmental characters of interest among algal Charophytes. Intron insertion and deletion events are considered to be evolutionarily conservative, making them useful for testing of hypothesized relationships based on other forms of data (Manhart and Palmer, 1990; Graham, 1993; Qui and Lee, 2000). Nuclear genes coding for actin are conserved and ubiquitous across eukaryotes. These features make actin gene base pair data useful for phylogenetic analyses (An et al., 1999). Beyond ribosomal RNA genes, the use of nuclear genes for phylogenetic analysis of the algal Charophyta has been extremely limited. Both sources of data, chloroplast introns and actin gene DNA sequences, support the relationships among algal charophytes found by Karol et al. (2001) and Turmel et al. (2002b).

3.4.3   Other Phylogenetic Inference

Investigation of tRNA-Ala and tRNA-Ile genes in the transcribed spacer region of chloroplast genomes has revealed a lack of interrupting introns in cyanobacteria, likely closely related to the symbiotically acquired primary plastids (Delwiche, 1997). Furthermore, these introns are missing from the tRNA genes examined in the chloroplast genomes of the algal Chlorophyta. New sequences produced in this study reveal the absence of both introns from the chloroplast tRNA genes of *Chlorokybus*. Recently published results (Turmel et al., 2002b) demonstrate that the tRNA-Ala gene is also interrupted by group II introns in members of the charophyte orders Klebsormidiales (*Klebsormidium*, *Entransia*), Zygnematales (*Cosmarium*, *Mesotaenium*, *Spirogyra*, *Staurastrum*, *Zygnema*), Coleochaetales

(*Coleochaete*, *Chaetosphaeridium*), and Charales (*Chara*, *Nitella*). In all of these taxa except genera of the Zygnematales an intron is also known to be present in the chloroplast tRNA-Ile gene (Turmel et al., 2002b). It is possible that a genome rearrangement event has affected the chloroplast DNA sequence in Zygnematales, as it is not clear that the transcribed spacer region organization demonstrated in each of the other orders is conserved in the algae of the Zygnematales. This distribution, introns present in the later-diverging lineages and the absent in both outgroup taxa and *Chlorokybus,* supports the placement of *Chlorokybus* near the base of the Charophyta clade.

3.5   Conclusions

Analyses of these new data support the view of evolutionary transitions presented in the literature review above. Character states found in the scaly biflagellate unicell, *Mesostigma*, are likely ancestral conditions among the charophytes. Branched filamentous thalli, like those in the Charales and Coleochaetales, including the genus *Chaetosphaeridium*, are likely derived conditions resulting from a set of advanced cellular and developmental capabilities possessed by algal lineages more closely related to the embryophytes. Continued testing of phylogenetic hypotheses will result in an even more refined understanding of the evolutionary relationships among algal members of the Charophyta. New observations of structural features, as well as discoveries of developmental mechanisms through the application of molecular genetics methodology, will add to the growing matrix of characters that can be mapped onto the tree of green life. This

perpetual process should lend to a cascade of increasingly precise hypotheses

explaining the evolution of this fascinating and important group, the Charophyta.

Appendices

Appendix A Isolation of total RNA from algal charophytes

These methods are based on the Sigma Tri Reagent protocol, and modified by the author according to Sambrook and Russell (2000) and experience gained while working on algae of the Charophyta.

1. Harvest thalli and remove excess culture medium. Record mass of the algal material.

2. To a 50-mL PPCO Oak Ridge centrifuge tube, add 1 mL of Sigma Tri Reagent per 50-100 mg thallus material. Close the tube and coat entire interior surface area of the tube by manual agitation followed by gentle rocking for ~5 minutes.

3. Add algal thallus material to tube containing Tri Reagent. Volume of algal material should not exceed 20% the volume of Tri Reagent. Homogenize thalli and Tri Reagent using the Polytron for ~1 minute at 80-100% power. Homogenized samples can be stored at -70°C for up to 1 month.

4. Allow sample to stand at room temperature for ~5 minutes. Centrifuge the homogenate at 12,000 x g at 4°C for 10 minutes. Transfer the translucent supernatant to a fresh tube. The supernatant contains RNA and protein. The pellet should contain high molecular weight DNA.

5. Add 0.2 mL pure chloroform per 1 mL of Tri Reagent used in step 2. Cover and shake the sample vigorously for 15 seconds. Allow the sample to stand at room temperature for 2-15 minutes.

6. Centrifuge the mixture at 12,000 x g at 4°C for 15 minutes. Separation should result in three phases—a colorless upper aqueous phase containing RNA, an interphase containing DNA, and a red organic phase containing protein. Interphase and organic phases can be stored temporarily at 4°C for subsequent isolation of DNA and protein, as per the Sigma Tri Reagent protocol.

7. Exercising care not to disturb the DNA-containing interphase, transfer the aqueous phase containing RNA to a fresh tube. For each 1 mL Tri Reagent used in step 2, add 0.25 mL isopropanol and 0.25 mL RNA Precipitation Solution. Mix thoroughly and allow the sample to stand at room temperature for 10 minutes.

8. Centrifuge the sample at 12,000 x g at 4°C for 10 minutes. Precipitated RNA will form a pellet on the side and bottom of the tube.

9. Remove supernatant and wash the RNA pellet by adding at least 1 mL 75% ethanol for each 1 mL Tri Reagent used in step 2. Vortex the sample and centrifuge at 7,500 x g at 4˚C for 5 minutes. RNA sample can be stored in the ethanol solution at 4˚C for up to 1 week and at -20˚C for up to one year.

10. Repeat the wash step described in step 9.

11. Remove ethanol from the pellet and allow to air dry on the bench top for 5-10 minutes. Do not allow the pellet to dry completely.

12. Resuspend the pellet in an appropriate RNase-free storage buffer, or resuspend in 1.5 mL Binding Buffer and proceed on to poly(A)+ selection protocol (Sambrook and Russell, 2000; Eric Haag, personal communication).

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